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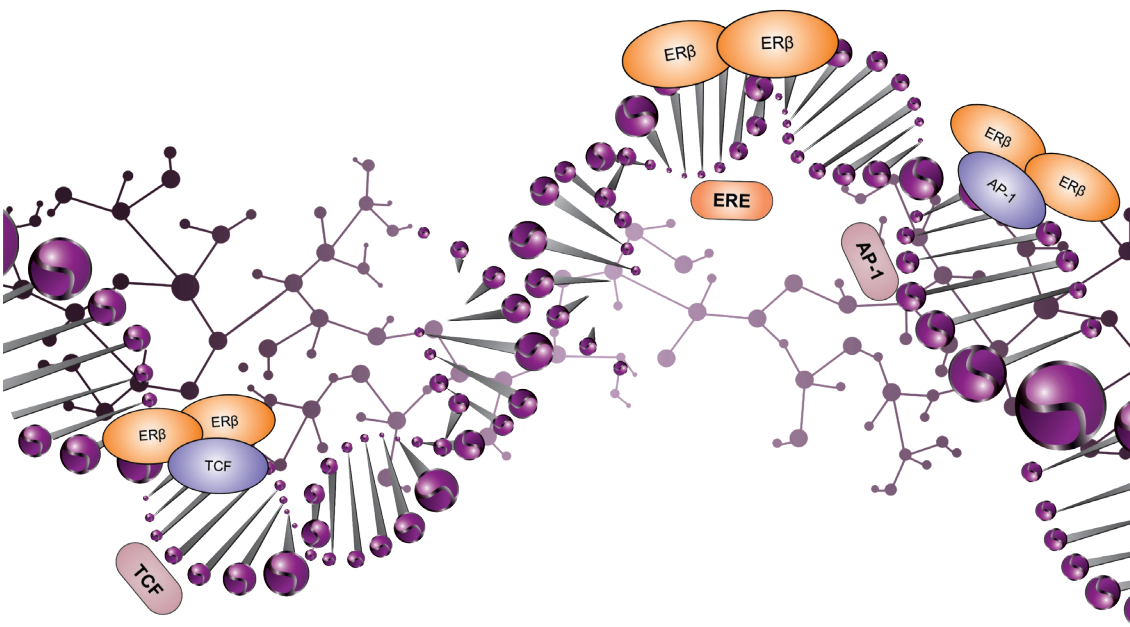
Estrogen receptor beta transcriptional regulation: A potential mechanism for colon cancer prevention



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By

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To my parents and
my father-in-law

ABSTRACT

Colorectal cancer (CRC) is the third leading cause of death from cancer in both men and women in the Western world. Improved screening efforts, surveillance, and treatment have reduced CRC mortality in older patients. However, the incidence is increasing in young adults, even in the absence of CRC family history. This may indicate an influence of increasing obesity, changed dietary patterns, and lifestyle factors. The progression of CRC is a multistep procedure that takes 10-15 years, thus offering a time to implement preventative measures and early detection. There is a critical need to develop more effective preventive therapies due to the risks posed by current prevention therapies. The best CRC prophylactic agent should be both safe and suitable to use for a long time (1).

In preclinical studies, estrogen has been shown to protect from CRC, and substantial evidence suggests it is through estrogen receptor beta (ERβ). Natural ERβ selective agonists have been tested in phase II clinical trials to treat menopause symptoms and proven to be safe and well-tolerated with no side effects (2, 3). Thus, selective activation of ERβ with selective agonists, which do not activate estrogen receptor alpha (ERα), is a potential clinical approach in preventing adenomatous polyps progression into CRC. However, the mechanism of these beneficial ERβ effects is not well understood, and there is a significant knowledge gap in this area.

The overall aim of this thesis was to identify the mechanistic background of the intestinal ERβ-mediated antitumorigenic effects in the colon and further to explore ERβ as a preventative approach in CRC. One specific aim was to determine whether ERβ present specifically in colon epithelium is responsible for protecting from CRC, which is addressed in Paper I. To understand the impact of ERβ in protecting from colitis-associated CRC (CA-CRC), we have induced colitis in intestinal-specific ERβ knockout mice of both sexes. The loss of intestinal ERβ aggravated CA-CRC in a sex-dependent manner. The incidence of tumors increased in males, while in females, the size of the tumors was enhanced. We identified that ERβ attenuates tumor necrosis factor alpha (TNFα) induced epithelial cell damage and modulates the regulation of key nuclear factor-κB (NFκB) members. As a direct consequence, ERβ was found to reduce inflammation and to control intestinal crypt cell proliferation.

Another aim was to explore transcriptional regulation by ERβ through mapping of chromatin binding sites and interaction with NFκB, which is studied in Paper II and IV. Commonly used ERβ antibodies have been shown to be unspecific towards ERβ; this study used a validated ERβ antibody to map genome-wide ERβ binding sites in colon cancer cells. We observed that the presence of ERβ also modulated the regulatory chromatin mark H3K27AC in potential enhancers of transcriptional regulation, Wnt signaling, and cell proliferation. Further, motif analysis indicated a novel ERβ colon-specific cross-talk with TCF, and KLF motifs supported a interaction between β-catenin/TCF and ERβ. We found that ERβ binds and regulates several important tumor suppressors and oncogenes in CRC cells, such as CST5 and LRP6, consistent with its proposed antitumorigenic activity. We also revealed the p65 cistrome in CRC cell lines

and showed that ERβ alters the p65 chromatin binding in a cell-type-dependent manner. We found that ERβ chromatin binding sites were enriched among circadian clock genes and also that ERβ modulates p65 binding to core clock genes in CRC cells, indicating potential cross-talk between ERβ and circadian clock gene regulation.

The final aim was to investigate the impact of ERβ, and estrogen signaling in high-fat diet (HFD) induced inflammation in colon, explored in paper III. We fed mice with an HFD for 13 weeks and treated them with estrogenic ligands for the last three weeks prior to sacrifice. The colon transcriptome showed predominant sex differences, and selective activation of ERβ reduced macrophage infiltration and epithelial cell proliferation induced by HFD. We demonstrated that ERβ opposes HFD-induced dysregulation of core circadian clock genes *in vivo*, further strengthening the role of ERβ in circadian rhythm.

Taken together, these results highlight the chemopreventive potential of ERβ in CRC in both sexes. The identified cross-talk with TNFα/NFκB pathway, Wnt signaling, regulating genes involved circadian clock, and tumorigenesis reflected ERβ protection/antitumor activity against CRC progression and development (as illustrated in Figure 1).

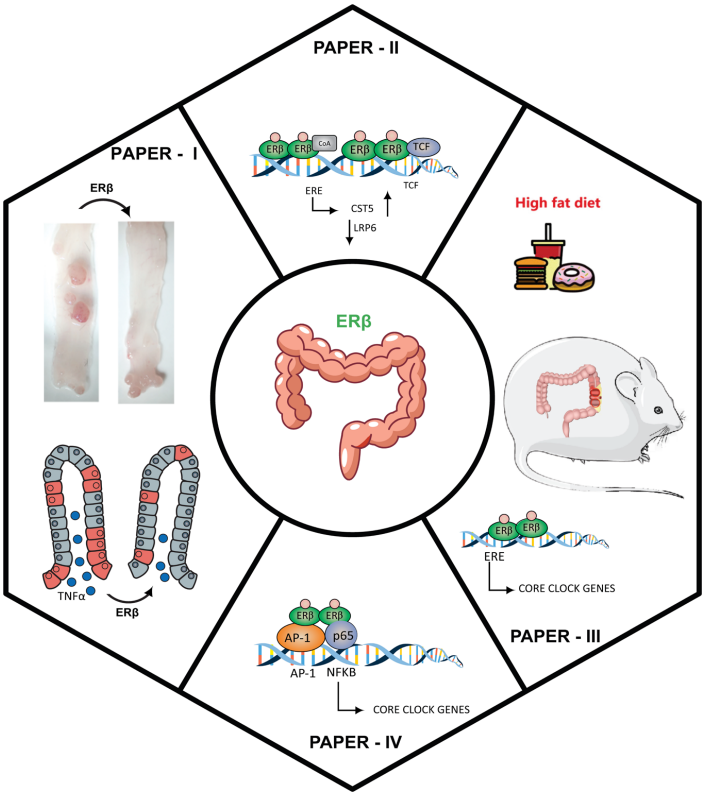

Figure 1. Schematic summary for the novel role of ERβ in the prevention of CRC.

LIST OF SCIENTIFIC PAPERS

I. Hases L, **Indukuri R**, Birgersson M, Nguyen-Vu T, Lozano R, Saxena A, Hartman, J, Frasor J, Gustafsson JÅ, Katajisto P, Archer A, Williams C. Intestinal estrogen receptor beta suppresses colon inflammation and tumorigenesis in both sexes. (2020) Cancer Letters. 492:54-62.

II. **Indukuri R**, Jafferali MH, Song D, Damdimopoulos A, Hases L, Zhao C, Archer A, Williams C. Genome-wide estrogen receptor β chromatin binding in human colon cancer cells reveals its tumor suppressor activity. (2021) International Journal of Cancer. 1-15.

III. Hases L, Archer A, **Indukuri R**, Birgersson M, Savva C, Korach-André M, Williams C. et al. High-fat diet and estrogen impacts the colon and its transcriptome in a sex-dependent manner. (2020) Scientific Reports. 10: 16160.

IV. **Indukuri R**, Hases L, Archer A, Williams C. Estrogen Receptor Beta Influences the Inflammatory p65 Cistrome in Colon Cancer Cells. (2021) Frontiers in Endocrinology. 12:650625.

CONTENTS

LIST OF ABBREVIATIONS

5-ASA	5-aminosalicylic acid
5-FU	5-fluorouracil
AF1/2	Activation function 1/2
AOM	Azoxymethane
AP-1/2	Activator protein 1/2
APC	Adenomatous polyposis coli
AR	Androgen receptor
ARNTL	Aryl hydrocarbon receptor nuclear translocator like
ATAC-Seq	Assay for Transposase-Accessible Chromatin using sequencing
BCL3	B-cell lymphoma 3-encoded protein
BIRC3	Baculoviral IAP repeat-containing 3
BMI	Body mass index
BRAF	V-RAF murine sarcoma viral oncogene homolog B1
Co A	Co activator
CCND1	Cyclin D1
CD	Control diet
CEA	Carcinoembryonic antigen
ChIP	Chromatin immunoprecipitation
ChIP-Seq	Chromatin immunoprecipitation followed by sequencing
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CK1	Casein kinase1
CMS	Consensus molecular subtypes
COX2	Cyclooxygenase2
CRC	Colorectal cancer
CREB	Cyclic AMP response element-binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CTNNB	Beta-catenin
CTSD	Cathepsin D

DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	DNA binding domain
DPN	Diarylpropionitrile
DSS	Dextran sodium sulfate
E1	Estrone
E2	17β-estradiol
E3	Estriol
E4	Estetrol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERE	Estrogen response element
ERα/β	Estrogen receptor α/β
ERβKOVil	ERβ knockout mice
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FEB	Fecal occult blood
FOXA	Forkhead box a
FOXM1	Forkhead box protein M1
FOXO	Forkhead box O
GATA	GATA binding protein
GPER1	G protein-coupled estrogen receptor 1
GSK	glycogen synthase kinase3
H3K27ac	Histone 3 lysine 27 Acetylation
H3K4me	Histone 3 lysine 4 methylation
H&E	hematoxylin and eosin
HDI	Human development index
HFD	High-fat diet
HIC	Chromosome conformation capture (HiC)
HRT	Hormone replacement therapy
IBD	Inflammatory bowel disease

IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
JUN	Jun Proto-Oncogene
KLF	Kruppel-like factor
KRAS	Kirsten rat sarcoma viral oncogene
LBD	Ligand binding domain
LDL	Low-density lipoprotein
LEF	Lymphoid enhancer factor
LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5
LRH1	Liver receptor homolog 1
LRP	Lipoprotein receptor-related protein
LRP5/6	Lipoprotein receptor-related protein 5/6
LXR	Liver X receptors
MAPK	Mitogen-activated protein kinase
MMR	Mismatch repair
MPA	Medroxyprogesterone acetate
mRNA	Messenger RNA
MSI	Microsatellite instability
MYB	Myeloblastosis viral oncogene
MYC	Master regulator of cell cycle entry and proliferative metabolism
NFκB	Nuclear factor-κB
NR	Nuclear receptor
NSAID	Non-steroidal anti-inflammatory drugs
NTD	N-terminal domain
PCR	Polymerase chain reaction
PER2	Period circadian protein homolog 2
PPAR	Peroxisome-proliferator-activated receptors
PPT	Propylpyrazoletriol
PROX1	Prospero homeobox 1
RAR	Retinoic acid receptors

RIME	Rapid immunoprecipitation mass spectrometry of endogenous proteins
ROS	Reactive oxygen species
RUNX	Runt-related transcription factor 1
RXR	Retinoid x receptor
SERM	Selective estrogen receptor modulator
SFREs	SF-1 response elements
SMAD	Mothers against decapentaplegic homolog 2
SP-1	Stimulating protein 1
SRC-1	Steroidal co-receptor activator 1
TCF	T cell factor
TF	Transcription factor
TGFβ	Transforming growth factor beta
TGFβR1/2	Transforming growth factor beta receptor 1/2
TNBS	Trinitrobenzene sulfonic acid
TNFα	Tumor necrosis factor α
TNM	Tumor-node-metastasis
TP53	Tumor protein p53
TRE	TPA response element
VDR	Vitamin d receptor
WNT	Wingless-type MMTV integration site family
WT	Wild type

1 INTRODUCTION

Globally, we have entered an era of increased living standards and affordable access to quality healthcare with better diagnosis methods and treatment of diseases. Life expectancy is also raised in most parts of the world as a result of access to health care. Improved medical treatments, however, have decreased death rates primarily from communicable diseases. By contrast, cancer-related deaths have increased by 40% over the past 40 years. A further 60% increase in cancer deaths was expected by 2030, with an estimated 13 million people dying of cancer, as reviewed in (4, 5). However, a recent study in the US population reported a 26% decline in the cancer death rates over two decades (6). Colorectal cancer (CRC) has become a leading cause of cancer, affecting 1.23 million patients each year and accounting for 10% of cancer-related deaths in Western countries (4, 5).

1.1 Colorectal cancer

CRC is defined as a tumor that originates from the colon and rectum. It is the third most prevalent cancer in the world, with an estimated 880 792 deaths each year, 1.8 million new cases, and 1.2 million deaths expected in the next ten years (7, 8). Roughly 66% of the cases are colon cancers, and the remaining are rectal cancers (9). After lung, breast, and prostate cancer, CRC is the fourth most common malignant tumor in terms of incidence, yet it is ranked the third in regards to cancer mortality (8).

1.1.1 Epidemiology

Colorectal cancer classifies as a symbol of socioeconomic growth, and, in nations experiencing significant development, prevalence rates are inclined to increase consistently with a high human development index (HDI) (10, 11). An upsurge in previously low-risk and lower HDI nations signifies lifestyle changes along with diets, such as changes toward an amplified consumption of animal-source diets, low physical activity, and body mass is independently correlated with CRC risk (12, 13).

For decades, CRC was considered an old age disease; however, recent data indicate increased CRC incidence in young adults under the age of 50 (14-16). Most of these individuals under the age of 50 would not meet the screening criteria. One recent study identified the rise of rectal cancers compared to colon predominantly in young individuals (14). The rising incidence in young birth cohorts indicates the influence of dietary patterns, excess body weight, and lifestyle factors (13).

1.1.2 Risk factors

CRC is a complex disease being influenced by multiple factors such as aging, chronic inflammation, poor dietary habits, obesity, and lifestyle. The most significant risk factor for CRC is older age; the disease is rare before age 40 (17). Around 80% of CRC cases are sporadic; only 10% are caused by genetic predisposition such as Familial adenomatous polyposis (FAP) and Lynch Syndrome. FAP is caused by inherited germline mutations in the

tumor suppressor gene adenomatous polyposis coli (APC). Lynch syndrome is characterized by mutations in mismatch repair genes (MMR) (18, 19).

Male gender increases the risk of CRC incidence and overall survival (17). Patients with inflammatory bowel disease and ulcerative colitis also have a higher risk of developing colon cancer (20). In CRC, substitution mutations that change single amino acids were the highest number of mutations indicating CRC's are most vulnerable to environmental factors (21). Thus, lifestyle-related diseases, such as metabolic syndrome, cause between 40-70% in terms of CRC development, and diet has been shown to influence CRC risk (22-24). A recent study indicated that high intakes of red meat and processed meat increase the risk of CRC (25), while phytoestrogens or isoflavones dietary intake provided a protective effect in CRC patients (26). High vitamin D levels are correlated with improved survival and decreased recurrence. Supplementary calcium intake was associated with reduced colon cancer risk (27, 28). In the UK population, using a modeling approach, 5% of diagnosed colon cancer cases were estimated due to low levels of physical activity. A meta-analysis showed that high physical activity reduces the overall risk of CRC by 24% compared to the least active individuals (29, 30).

1.1.3 Tumor staging and subtypes

In colorectal cancer, the tumor's pathological stage is generally considered the most critical determinant of outcome. The TNM classification is the most widely used, which considers tumor invasion, spread to lymph nodes, and metastasis to stage CRCs (31). The high degree of heterogeneity in CRC, at both genomic and transcriptomic levels, leads to further categories based on gene expression profiles.

Consensus Molecular Subtypes (CMS) classification identified four subtypes (CMS1-CMS4) and helped in personalizing oncological care by distinguishing the patients who may benefit from adjuvant chemotherapy (32). The CMS1 subtype is associated with major histological changes, more often seen in women, and is linked to MMR status. The tumors of the CMS2 are the most diverse among all, whereas the CMS3 subgroup contains a high incidence of KRAS mutations. The CMS4 group is considered a pro-inflammatory type due to increased mesenchymal and stromal genes instead of genes involved in cancer, indicating the importance of inflammation in CRC. The CMS4 group had the most advanced stages of CRC of all the groups, as well as the highest tumor budding compared to the CSM2, CSM3 subtypes(33).

1.1.4 Diagnosis and treatments

CRC's routine diagnosis is based on physical examinations, colonoscopy, and computer tomography colonography. The serum tumor marker carcinoembryonic antigen (CEA) or fecal occult blood is also measured using laboratory tests (FEB). In most of Europe, Canada, and North America, screening initiatives testing FEB and using colonoscopy are currently in progress to detect CRC early (34). A CRC screening program started in Stockholm in 2008 and

within five years identified 0.1 percent with CRC out of 1.8 percent tested with a colonoscopy (35).

Studies have been conducted using non-steroidal anti-inflammatory drugs (NSAIDs) to prevent CRC. NSAIDs such as aspirin and celecoxib inhibit the cyclooxygenase-2 (COX-2) enzyme, which induces inflammation and reduces the risk of colon cancer. Two extensive clinical studies revealed that long-term use of NSAIDs reduces CRC incidence by 37% (36, 37). However, these clinical trials stopped early due to cardiac side effects of COX-2 inhibitors such as celecoxib and rofecoxib (17, 38, 39). A randomized control trial of 164,225 patients confirmed that aspirin increased the risk of bleeding (40). Hence COX-2 inhibitors, which had previously been approved by the European Union (41) and the United States (42), were no longer used to prevent CRC, and there is a need for developing new and safe targets.

The cure for colorectal cancer is determined by the degree to which cancer has spread. Complete surgical resection is the only approach in treating colorectal cancer in stages between I and III. Primarily, 2 out of 3 individuals who go through surgical resection will have cancer regrowth or metastases. Thus, to treat and prevent a recurrence, chemotherapy treatment with single drug 5-Fluorouracil (5-FU) or in combination with oxaliplatin, capecitabine used (43).

1.2 Pathways involved in CRC

Notably, the three common pathways in CRC development are chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP). Primarily, the CIN pathway accounts for 85% of the sporadic CRCs. It is mainly caused by chromosomal imbalance and loss of function mutations in tumor suppressor genes. Fundamentally, MSI is categorized by mutations in MMR genes regulating microsatellites in DNA replication (44). MSI creates frameshift mutations that inactivate genes involved in the MMR process (45). Aberrant methylation of CpG islands leads to epigenetic instability in CRC, and 10-20% CRCs contain a high percentage of methylated CpG loci (46). Nonetheless, the mechanisms leading to CIMP are still unknown.

1.2.1 Molecular pathogenesis

The evolution of colon cancer is mainly due to the progressive accumulation of genetic mutations through which cells acquire the ability to sustain cancer hallmarks. The six biological hallmarks of cancer are proliferative signaling, inhibiting growth suppressors, resisting cell death, evading apoptosis, and inducing angiogenesis. Genetic and epigenetic alterations activate oncogenes and inactivate tumor suppressor genes to enable cancer hallmarks (47). Aberrant colonic crypts drive the normal colonic epithelium into adenomatous polyps, benign tumors with malignant potency. Benign adenomas develop into carcinomas over 10-15 years due to the accumulation of genetic mutations primarily in adenomatous polyposis coli (APC), KRAS proto-oncogene, and tumor protein p53 (TP53) genes. The pathways involved in the molecular progression of CRC are depicted in figure 2 and discussed as follows.

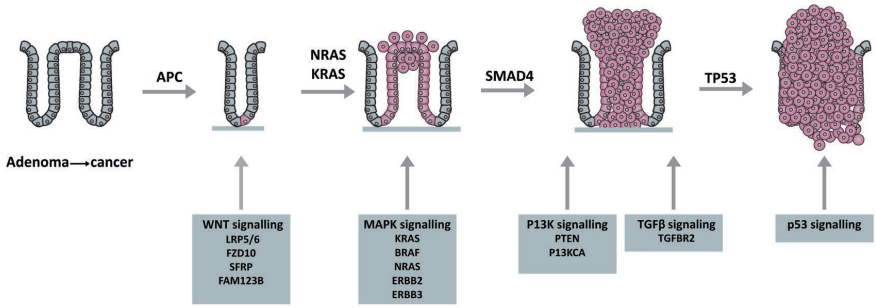

Figure 2: Molecular progression of colorectal cancer. Inspired by Kuipers et al., 2015.

1.2.2 Wnt/β-catenin signaling pathway

Wnt signaling is a central mechanism that controls cell fate decisions which mainly acts through frizzled protein, low-density lipoprotein receptor-related protein 6 (LRP6) receptors. In a normal colon, the Wnt ligand maintains stem cell homeostasis, and the Wnt signaling target gene Lgr5 is a well-established marker for intestinal stem cells (48). CRC cells with high expression of Lgr5 clonally expand and form colonies compared to cells without Lgr5 expression (49). High Wnt activity was observed in colon cancer stem cells and cancer stemness regulation (50).

Wnt signaling is divided into two pathways, canonical, dependent on the β-catenin and, non-canonical pathway, independent of β-catenin. β-catenin is encoded by the gene CTNNB1 and plays a vital role in cell morphology. Both cytoplasmic and nuclear β-catenin levels are strictly regulated by a group of proteins called β-catenin destruction complex, which keeps the levels of β-catenin low in the absence of a Wnt signal. This complex is formed by scaffolding protein axin, glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1), and APC protein. APC is a tumor suppressor gene that negatively regulates β-catenin and controls proliferation, differentiation, and migration promoted by Wnt signaling. APC is mutated in more than 80% of CRC patients (51). When Wnt binds to the frizzled receptors and LRP5/LRP6 in the canonical pathway, it inactivates the β-catenin destruction complex and activates β-catenin. β-catenin, which does not possess a DNA-binding domain, interacts with TCF (T- cell factor) or LEF (lymphoid enhancer factor) transcription factors to regulate Wnt target genes involved in cell migration, proliferation, and survival such as CCND1 (cyclin D1), PTGS2 (COX-2) and MYC (c-Myc) (52).

In the absence of a Wnt signal, β-catenin destruction complex phosphorylates β-catenin, while APC constantly ubiquitinates and degrades the β-catenin. The central region of the APC protein mainly coordinates with β-catenin through seven 20 amino acid repeats. APC mutations in colon cancer were primarily found in the third 20 amino acid repeat of the protein, which has been shown to be the most highly phosphorylated site with an increased affinity for β-catenin (53). APC gene inactivation fails to degrade β-catenin. β-catenin then increases transcription through TCF (Figure 3). Inactivating APC gene mutations initiate the majority of CRCs by

increasing Wnt signaling. Mutations of the APC gene have been reported as an early CRC mutation for the acquisition of cancer hallmark inactivated tumor suppressor genes (54). In FAP patient's germline APC mutations causes hundreds of polyps in the colon and rectum (18).

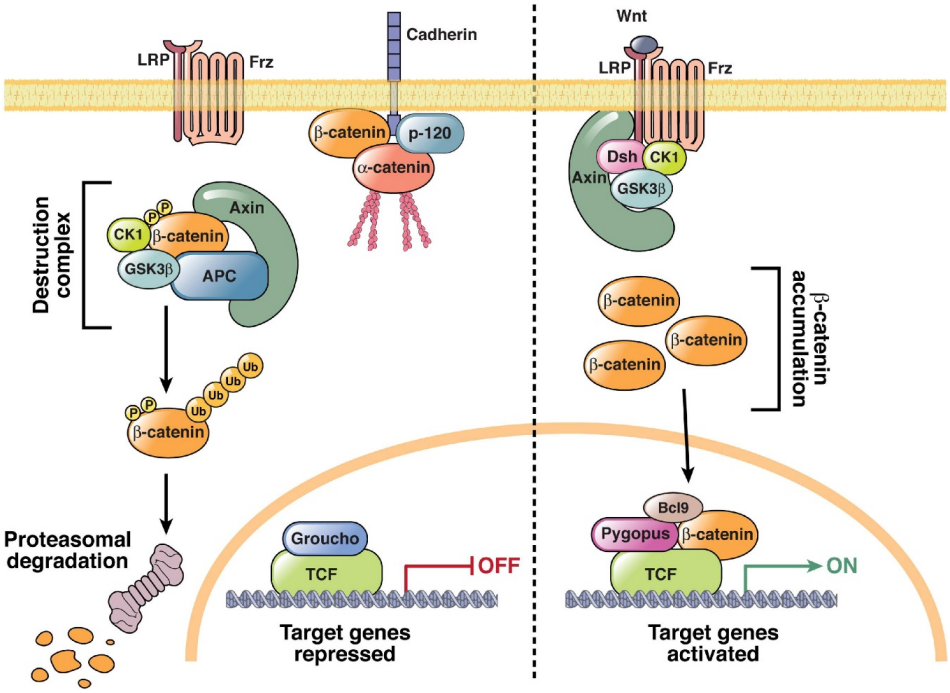

Figure 3. Wnt signaling pathway in colorectal cancer (Pino et al. Gastroenterology 2010, permission obtained to print 5055420182095)

1.2.3 RAS/MAPK pathway

Adenoma to carcinoma progression is initially influenced by the KRAS gene, a proto-oncogene encoding for an ATPase protein that transmits an extracellular signal through MAPK and enhances cell proliferation. Activating mutations in the KRAS gene permanently activates the protein to sustain proliferative signaling and avoid apoptosis. Single point mutations in codon 12 and 13 lead to an oncogenic phenotype and are hotspot mutations in the KRAS gene (55).

1.2.4 p53 pathway

p53 is a tumor suppressor that regulates cell cycle arrest and apoptosis. Inactivating p53 mutation is a common and critical molecular step in CRC progression. Loss-of-function p53 mutations are most common (56, 57) and result in proliferative activity and resistance to cell death. Unlike mutations of the APC and KRAS genes, and unlike some other cancers (such as non-melanoma skin cancers), p53 mutations usually occur in the later stages of colorectal carcinogenesis.

1.2.5 TGF-β pathway

TGF-β is a member of the cytokine family regulating epithelial systems and inflammatory processes, particularly in the gut. TGF-β receptors (TGFBR1, TGFBR2) phosphorylate SMAD2 and SMAD3, which then form a complex with SMAD4 and move to the nucleus to regulate transcriptional activity. TGF-β also maintains tissue homeostasis and regulates cell proliferation, adhesion, and survival. Cancer cells escape this pathway during malignant evolution through mutations that truncate TGF-β receptors and inactivate the SMAD4 gene (58, 59).

1.2.6 TNFα/NFκB signaling

The function of the nuclear factor of κB (NFκB) was previously thought to be immune-related, but oncogenic mutations activated NFκB in lymphoid cancers have revealed its important role in cancer. NFκB is a family of transcription factors, which regulate innate immune and inflammatory responses. A constitutive expression of NFκB in cancers revealed other functions of NFκB, such as cell proliferation, migration, and apoptosis (60, 61). There is a large amount of evidence suggesting NFκB participation in carcinogenesis by activating cellular gene expression. To date, five different NFκB family members have been identified; NFκB1 (p50/p105), NFκB2 (p52/p100), RelA (p65), RelB, and c-Rel. NFκB protein dimers bind to the promoter of target genes on a standard specific sequence known as "κB site." The term NFκB mostly refers to p65-p50 heterodimer, which is the primary dimer in most cell types. NFκB transcriptional activity is dependent mainly on p65, which contains the most potent transcriptional active domain (61-64).

1.3 Colorectal cancer - preventable cancer?

As described above, the origins and causes of colorectal cancer (CRC) are many. Many risk factors are modifiable (17), which allows the potential prevention of most CRCs development (22-24).

1.3.1 Inflammation

Inflammation is one of the most important hallmarks of cancer, promoting cancer development and progression through different mechanisms (65). Inflammatory diseases such as colitis, pancreatitis, and hepatitis, have been associated with an increased risk of colon, pancreatic, and liver cancers, respectively. A prolonged inflammatory condition attracts macrophages, leukocytes which secrete tumor necrosis factor-alpha (TNFα), and reactive oxygen species (ROS). This aggravates DNA damage (66) and results in mutations (67, 68). Further, chronic inflammation profoundly affects metastasis through epithelial-mesenchymal transition (EMT), providing mobility and the ability for cells to migrate from the primary tissue (69). Through activation of wound healing processes, the inflammatory response augments angiogenesis and invasion, advancing the tumors' development (70).

As an inhibitor of cyclooxygenase-2 (COX-2), a component that ranks as an important mediator of the inflammatory reaction, Aspirin was shown to be substantially effective in preventing adenomatous polyp and CRC (71, 72). However, Aspirin is not advisable because of the increased risk of bleeding. Frequent usage of Non-Steroidal Anti-Inflammatory Drugs (NSAID) is associated with a lower risk of CRC (71, 72), even though the case is different when there is an occurrence of BRAF mutation-caused CRC (73). However, trials with NSAID celecoxib have ceased due to cardiac side effects (38, 40). New treatments are necessary due to the risks posed by current prevention therapies.

1.3.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) results from the chronic inflammation of the intestine triggered by environmental and immunoregulatory factors, unbalanced gut microbiota, and genetic predisposition. IBD has increased rapidly worldwide and also, 25% of the detected cases were below the age of 20, indicating a rise in children and adolescents (74). IBD is categorized into Crohn's disease and ulcerative colitis. In many cases, Crohn's disease has its effects on the entire GI tract, whereas ulcerative colitis can be termed as a disease of the large intestines, specifically the sigmoid and rectal parts (75). IBD is a chronic condition that can be clinically managed using 5-aminosalicylic acid (5-ASA), doses of immunomodulatory (azathioprine), and anti -TNFα drugs such as infliximab. Nonetheless, surgery may be preferred in certain cases (74).

IBD increases the risk of CRC, and eventually, 20-30% of IBD patients will develop colitis-related colorectal cancer (CA-CRC) (76, 77). CA-CRC shows a complex malignancy by acquiring p53 mutations at an early stage compared to the sporadic type of CRC, where APC mutations are the first events (78). Sex differences were identified in CRC development in patients with IBD; men with IBD are at 60% increased risk to develop CRC compared to women (20).

1.3.3 Obesity and diet

Epidemiological evidence suggests that having a high Body Mass Index BMI (BMI) raises the risk of developing CRC (79). A large cohort study conducted among ~80,000 females identified that being overweight was connected to an increased risk of early-onset CRC in women. The foremost aspects of obesity are chronic low-grade inflammation and excess adipose tissue, which enhances the release of free fatty acids, interrupting insulin signaling in the skeletal muscle (80). Also, HFD is illustrated to affect intestinal permeability, microbiota, along with inflammation (81-83). A new study suggests that high-fat diet advances CRC development upsets the balance of bile acids within the intestine, activates a hormonal signal that helps cancerous cells thrive. In this case, such findings could clarify why colorectal cancer, a disease that can take years to develop, is becoming more common in younger people, following current trends indicating that higher-fat diets are more prevalent. (84). Consumption of an HFD causes metabolic dysfunction by disrupting the systemically harmonized circadian rhythm (85). For instance, a high-fat diet modifies the food intake of rats with extra food intake

during the day (86). It disrupts the rhythmicity of *Clock, Bmal1*, and *Per2* within the liver as well as the adipose tissue (87) (88). In the gut, circadian rhythm plays an essential role in regulating intestinal permeability, cell proliferation, gut microbiota, and metabolism, and perturbation of this fine balance can result in IBD and CRC (as reviewed in (89)). The dysregulation of circadian genes results in increased pro-inflammatory cytokines plus the body adiposity regarding the diet-induced obese mice (86). Indeed, meta-analyses have discovered a relationship between night shift work, obesity, and augmented CRC risk (90, 91).

1.3.4 Sex and hormonal factors

There are apparent sex differences in CRC. Compared to women, the prevalence of CRC is significantly higher in men. More aggressive proximal CRC is most common in women, while men predominantly suffer from distal CRC (as reviewed in (92)). Studies have been conducted to showcase the varying origins and causes of both distal and proximal CRC. Men and women have different gastrointestinal transit times, impacting the interaction between anti-carcinogenic, carcinogenic agents and colonic mucosa. Abnormal glucose metabolism promotes the growth and progression of CRC as reviewed in (93, 94), and sex differences highly influence glucose metabolism and DNA methylation patterns which alter insulin production (95). Sex hormones also play an essential role in the progression of certain cancers. Estrogens also impact gastrointestinal tract diseases (96, 97), including gastroesophageal reflux disease (98), irritable bowel syndrome, and IBD (99).

1.4 Estrogens in CRC

1.4.1 Estrogens

Estrogens are steroid hormones that are regarded as necessary for sexual and reproductive development in women and men. Estrogens are involved in various physiological functions within the female body, such as bone integrity, muscle mass, subcutaneous visceral fat, circadian rhythm, and homeostasis (100, 101). In men, estrogens are also critical during the development of their reproductive organs and sex differentiation of the brain (102).

The term estrogen denotes several chemically similar hormones within this group which include estrone (E1), 17β-estradiol (E2), estriol (E3), and estetrol (E4) (103). E2 is the most predominant circulating form of estrogen in pre-menopausal women and is 10 times more potent than E1 and 80 times more potent than E3 (104). The primary production of E2 is in the ovaries of premenopausal women. It is synthesized in the granulosa cells of the ovaries through a process called steroidogenesis. Dietary cholesterol, particularly low-density lipoprotein (LDL)-cholesterol, is the primary substrate for steroid hormone biosynthesis (103). Estrogens are a result of the action of aromatase, responsible for the conversion of testosterone to estradiol and androstenedione to estrone. The granulosa cells of the ovarian follicles and the corpora lutea produce E2. The synthesis of estrogens also occurs in smaller amounts in other tissues that express aromatase, such as the adrenal glands, liver, pancreas, adipose tissue, and breast (106).

In males, E2 is produced through aromatization in Sertoli cells, Leydig cells, and mature spermatocytes of the reproductive tract (102). In men and postmenopausal women, E1 is the most prominent estrogen and comes from a local synthesis of estrogens in extra gonadal target tissues by aromatization (105). Through this, the E1 may also be converted into E2 in peripheral tissues. These include adipose and breast tissue, vascular endothelium, smooth muscle cells, brain tissue, and bone cells (105, 107). The estrogens local production implies the signaling modality from endocrine to act locally as a paracrine or intracrine factor (105). E3 and E4 are only synthesized during pregnancy by the placenta and fetal liver, respectively (108, 109). All estrogens can bind to nuclear and membrane estrogen receptors, but their affinity and strength of the response vary. The most potent among all estrogens in inducing cell proliferation is E2, followed by E1 and E3 (110).

A recent study conducted using gastric biopsies found that parietal cells produce large amounts of estrogens within gastric mucosa and eventually secrete them to the portal vein (111). It is hypothesized that gastric E2 binds to the ER and controls certain functions or is metabolized into inactive estrogens by liver (112). Thus, it explains the pathophysiological importance of estrogens in gastrointestinal tract.

1.4.2 Estrogen receptors alpha and beta

Estrogen receptors (ERs) are the mediators of estrogens (102, 103). Estrogen mainly acts through three receptors, estrogen receptor alpha (NR3A1/ESR1), estrogen receptor beta (NR3A2/ESR2), and G protein-coupled estrogen receptor (GPER1, see below). ERα and ERβ are nuclear receptors. Nuclear receptors (NRs) are multifunctional proteins transducing signals of their cognate ligands. Humans have 48 nuclear receptors, including steroid, thyroid, vitamin D, and retinoid receptors. These are involved in signal transduction and act as ligand-inducing transcriptional regulators which control the activity of specific gene networks. They can be divided into three categories: endocrine NRs, orphan NRs and adopted NRs based on ligand binding. Endocrine NRs rely on hormones as their endogenous ligands, such as ERs. Orphan NRs have no known natural ligand, while adopted NRs have their natural ligand discovered after previously being considered orphans (113). The various forms of NRs include monomers (steroidogenic factor-1 (SF-1), estrogen-related receptor β) (114) and homodimers (ERs, and androgen receptors (AR), heterodimers with other nuclear receptors (retinoid X receptor (RXR), thyroid receptor, and vitamin D receptor (VDR), among others (115).

The most researched NR functions are the ones of AR in the prostate and ER in the breast. AR is a steroid receptor for testosterone which is a critical driver in prostate cancer, and several anti-androgen drugs are in use to reduce AR activity (116). ERα initiated and proliferate breast cancer, and a majority of the breast cancers contain ERα expression and responds to hormone treatment (117). About 70% of NR binding sites were prominent in open chromatin (113, 118). Nevertheless, NRs recruit a variety of chromatin-modifying factors to facilitate binding to target sites within closed chromatin.

HNF4, ER, LRH1, PPAR, VDR, RXR, RAR and LXR are the nuclear receptors expressed in the colon (113, 119). In both mouse and human models of CRC, the enteric NR transcriptome is downregulated during tissues' progression from normal intestinal epithelia to dysplastic lesions. Therefore, a therapeutic, diagnostic potential is proposed for these transcription factors in CRC (120, 121).

ERα was discovered in 1968 (122), and after 27 years, the second distinct estrogen receptor ERβ was identified in rat prostrate by the Gustafsson group. The ERα and ERβ genes are located on different chromosomes (6q25.1 and 14q22-24) (123-125). ERα is mainly expressed in the endometrium, ovary, mammary gland, bone, uterus, smooth muscles, and epididymis within the human body. On the other hand, ERβ can be found in the ovaries, testis, adrenal gland, and spleen (126).

1.4.3 ER structure

All nuclear receptor proteins are composed of a variable N-terminal domain (NTD), a DNA binding domain (DBD), a hinge region, a conserved ligand-binding domain (LBD), and a variable C-terminal domain. The DNA binding domain has two zinc finger motifs that serve as hooks and facilitate binding to chromatin in the nucleus. Each class has its own DNA binding recognition sequences, which is a distinguishing feature. Nuclear receptor ligand-binding domains are functionally quite similar, but their specificity and affinity for particular ligands differ (127).

Estrogen receptors are composed of five functional domains. Beginning from NH_2- to COO-terminus, Activation function 1 (AF1) and NH2 terminal domain (NTD) forms domain A/B, DNA binding domain (DBD) forms domain C, domain D is hinge region, ligand binding domain (LBD), and Activation function 2 (AF2) located within domain E and domain F in C-terminal of protein.

The NTD/AF1 domain is capable of ligand independent activity through phosphorylation and subsequent transcriptional activation of target genes (128, 129). DBD recognizes specific DNA sequences denoted as estrogen response elements (ERE) in target genes, facilitating ER binding to stimulate or repress the transcription of these genes (130). LBD/AF2 domain contains a hormone-binding pocket that facilitates ligand binding and dimerization. The orientation of the AF2 domain determines the functions activated by ligands as an agonist or antagonist (131). Besides, AF1 and AF2 are important regions that activate transcription and interact with other nuclear receptors or co-activators (132). The DBD and LBD are connected by the D domain, which includes amino acids that are important for post-translational modification (133). Additionally, the F domain controls the transcription of genes in a cell specific and ligand independent way (134, 135).

DNA-binding domains of ERα and ERβ are highly conserved, but the two nuclear receptors differ significantly in their N-terminal domains and in their ligand-binding domains. At hinge regions of ERα and ERβ, they share only 36% homology (124). Although both receptors

strongly bind estrogen, the overall homology of their ligand-binding domains is less than 55%, which has enables the development of receptor-selective ligands (124, 134, 136).

1.4.4 ERβ splice variants

ESR2 contains eight encoding exons, which spans 254 kb, and it has several isoforms due to alternative splicing of the last coding exon (137). These isoforms are modified in the 3' end of hERβ1(wild type ERβ) (Figure 4). Of all, wild-type ERβ (530 aa) is the only isoform that binds to the ligand (7). ERβcx can heterodimerize with ERα, hence widely seen as a dominant-negative receptor for ERα (138, 139). It has since been established in a recent study that ERβ splice variants ERβcx and ERβ5 are present in granulosa cell tumors (140). The study, however, does not clearly outline their roles in the pathogenesis and progression of cancer (137, 140).

There is evolutionary conservation of ERs between species (141). Rodents have two known ERβ splice variants: a full-length rat and mouse ERβ WT isoforms (549 amino acids), with a sequence similarity of 99% (131) and both mouse and rat ERβ2 isoforms with an 18-amino acid insertion in the LBD that leads to decreased ligand binding affinity of estrogen (142-144).

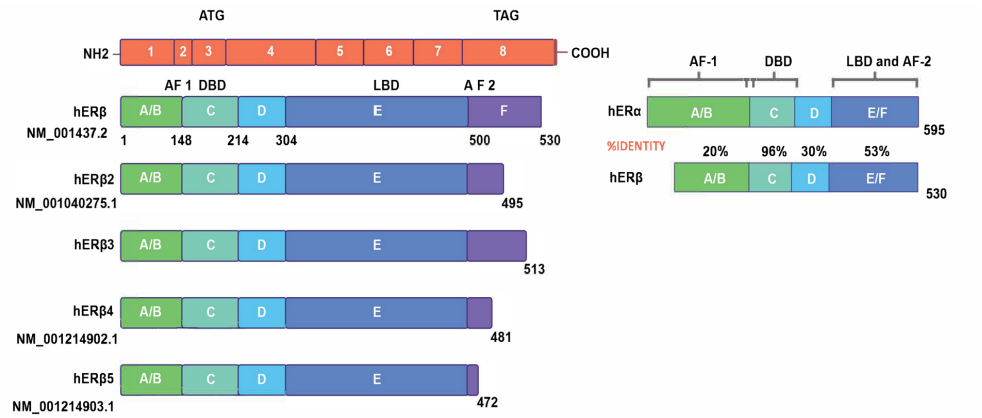

Figure 4. Estrogen receptor beta contains five domains, and all splice variants of ERβ identified in humans vary in F domain and the structural similarities between ERα and ERβ.

1.4.5 GPER1

The third estrogen receptor is GPER1 (G protein-coupled receptor 1) (145). This receptor does not share structural similarities with ERα or ERβ and is not a transcription factor (146, 147). The G-protein coupled receptors (GPCRs) family entails the biggest cell surface receptor and contains seven transmembrane domains (148). Compared to other estrogen receptors, GPER1 has a poor binding affinity to ligand E2 (17β-estradiol) and requires high levels of ligand to become activated (149, 150). However, once activated by E2, GPER1 produces rapid responses and activates intracellular signaling cascades by increasing cAMP production (151) and intracellular calcium release (149). Specifically, estrogen activated GPER1 is involved in

various physiological functions in human tissues and organs, such as nervous, immune, reproductive, cardiovascular mechanisms, and cancer development and metastasis (152). The rest of this thesis will not focus on GPER1, and ER will refer to ERα and ERβ.

1.4.6 Ligands

Estrogen is an endogenous ligand that activates ERs. Nevertheless, numerous other compounds presenting estrogenic effects that structurally or functionally resembles mammalian estrogens are recognized. Such compounds include phytoestrogens (naturally synthesized by plants) (153) and xenoestrogens (synthetic chemical compounds) from medicinal drugs, food additives, and plastics or environmental contaminants (154-156). Phytoestrogens can activate both ER's. Some phytoestrogens such as genistein, coumestrol, and liquiritigenin are more selective to ERβ based on their capability to bind to LBD with a higher affinity in comparison to that of ERα (157-159).

Whether natural or synthetic, ligands may be categorized as selective estrogen receptor modulators (SERMs), agonists, or antagonists. Agonistic ligands bind to ERs and trigger them, whereas antagonists block ERs activity upon binding. Agonists and antagonists bind at the exact position of the ERs LBD with different binding alignments. Agonist rearranges the ligand-binding domain conformation, creating helix 12 to rotate in a way to enable the free recruitment of co-activators while at the same time removing the co-repressors. By contrast, the antagonist disturbs helix 12 conformations to hinder association with co-activators (160, 161).

SERMs are a collection of synthetic nonsteroidal compounds that act as both agonists and antagonists of ERs, depending on the tissue type or estrogen receptor type. SERMs display antagonism in breast and uterus and a more agonistic nature in bones and liver (162). For this reason, they are utilized for the management of ERα-positive breast cancer (antagonism) and the deterrence of osteoporosis after menopause (agonism). A classic example is tamoxifen which acts as an antagonist in breast but as an agonist in uterus (163). Consequently, even though tamoxifen is the favored drug for treating ER-positive breast cancer, it can also prompt endometrial cell development and increase endometrial cancer risk (164, 165).

Another group of SERMs binds to only one of the ER subtypes. The ERα-selective agonist 4,4',4"-(4-propyl-1H-pyrazole-1,3,5-triyl) trisphenol (PPT) and the ERβ-selective agonist diarylpropionitrile (DPN) were used in the studies presented in this thesis. Notably, PPT binds to ERα with a 410-fold selectivity over ERβ (166), whereas DPN binds to ERβ with 70-fold selectivity over ERα (167).

To circumvent the classic actions of estrogens, such as stimulation of uterine proliferation, pathway-selective estrogen receptor (ER) ligands such as WAY-169916 were developed (168). WAY-169916 has shown strong anti-inflammatory activity in synoviocytes obtained from arthritis patients (169). Numerous other non-estrogenic compounds like androstane-3β and

17β-diol (3βD) have been shown to interact with the ERs (170, 171). One recent study also identified ketamine and its metabolites as a regulator of ERα (172).

1.4.7 ER Signaling pathways

ERs trigger transcriptional procedures and signaling events through direct binding to estrogen response element (ERE) in gene promoters or enhancers (genomic effects) or through processes that do not comprise a direct binding to the DNA (non-genomic effects), Figure 5 (103).

Direct genomic signaling: ERE-dependent nuclear signaling

In the classical pathway, ligand-activated ERs translocate into the nucleus, dimerizes, and binds directly to the consensus ERE sequence or estrogen half-sites. ERE is a 13-bp palindrome with a 3-bp spacer (5'-GGTCAnnnTGACC-3'), and ERE half-sites are either the proximal or distal half of the palindrome (173, 174). However, certain estrogen-responsive genes comprise EREs that diverge from the consensus through one or more nucleotides as well as being less potent regulators of the transcription than the consensus ERE (175). Nevertheless, regarding half ERE sites 5′-TGGGCTCA-3′, controversy arises about whether ER can bind to these sites (176). A study that incorporated genetic deletions within the ER-bound enhancers by utilizing CRISPR in MCF7 cells showed that two enhancers containing half EREs could not compensate for the full ERE site and indicate that the position of full ERE immensely affects the enhancer activity (177).

It can be noted that homodimers (ERα-ERα or ERβ-ERβ) and heterodimers (ERα-ERβ) can be formed by ERs based on the tissue type and ligand. Even though ERα and ERβ selectively bind to EREs with similar affinity *in vitro*, the mechanism and degree of transcription that the ERs control significantly differs via the ERE-dependent signaling pathway (173, 178). *In vitro* gel shift assays have shown that compared to ERα, ERβ has lower binding to non-consensus EREs, which account for most estrogen-responsive elements (e.g., FOS, JUN, pS2, CTSD) (179).

Indirect genomic signaling: ERE-independent nuclear signaling

Unlike the classical pathway, countless ER binding sites lack ERE components (180-182); nonetheless, ER's control the transcription of these genes through an approach called transcription factor cross-talk (183-186). As stated by the most current studies, a projected 35% of genes that are targeted by estrogen do not contain EREs (103). Previous studies (187-190) have revealed the potential of ER to coact or tether with a range of coactivators, for instance, activating protein-1 (AP-1), stimulating protein 1 (Sp1), RUNX1, and NFκB. A cross-talk between ERα and NFκB has been studied. A few studies (191, 192) report that ERα represses NFκB activity, but on the contrary, other studies report that ERα enhances NFκB activity (193, 194) using the same cell lines.

The most widely studied ER coactivator is AP-1. ER bind members of AP-1 complex (Jun/Fos heterodimer) transcription factors. Indeed, mapping ER binding sites across the genome shows much overlap with AP-1 binding sites (195). In cell lines, when treated with E2, ERα and ERβ

showed opposite transactivation of the AP-1 site: E2 activated transcription via ERα, while inhibited via ERβ (196). This was also noted in cyclin D1 gene expression, where E2-bound ERβ reduced expression of cyclin D1 and further blocked ERα-mediated activation of cyclin D1, as reviewed in (103).

Additionally, several estrogen-responsive genes lacking full EREs have ERE half-sites or binding elements for the orphan nuclear hormone receptor SF-1 [SF-1 response elements (SFREs)], serving as direct ER binding mechanisms (197). Several studies indicate that ERα, but not ERβ, binds to SFREs (198) and transcriptionally stimulates the SP1 component (199). Notably, studies point out many dissimilarities in ERβ and ERα coordinating with cofactors.

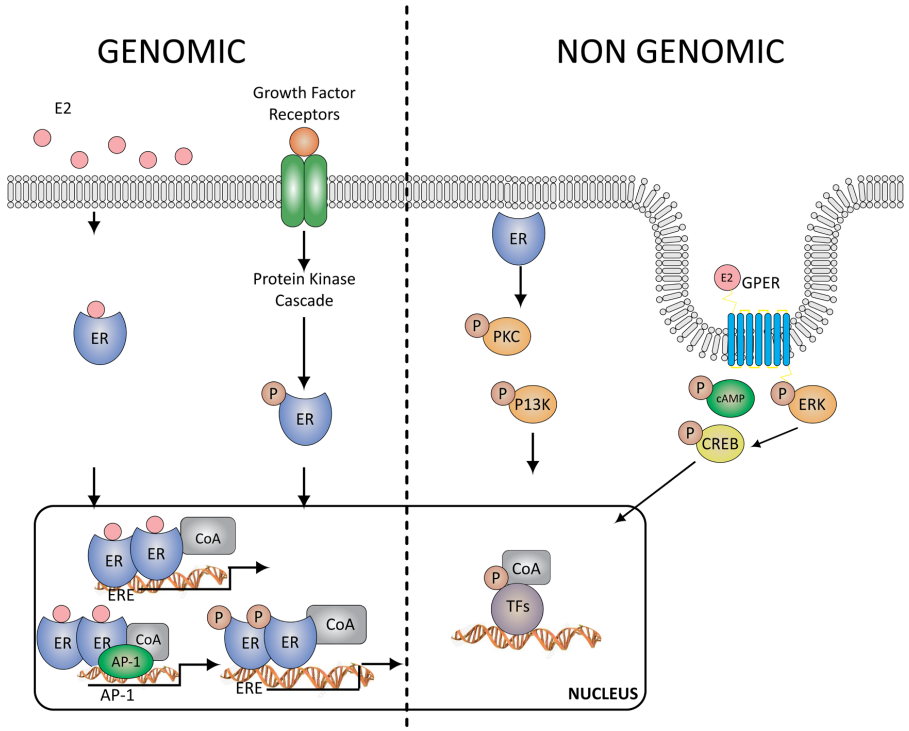

Figure 5. Different modes of ER signaling, including genomic, nongenomic, and signaling through growth factors. Inspired by Morselli et al., 2018.

Indirect signaling: membrane receptors, mitochondrial events, signaling molecules

The ERs exert cell-specific immediate effects by activating signaling molecules such as the Ras/MAPK pathway and cAMP. Activated kinases phosphorylate the ERs in the N-terminal domain, causing receptor dimerization, binding to DNA, and initiation of gene expression in a ligand independent fashion (131, 200). Furthermore, estrogen that binds to GPER1 also stimulates estrogen-dependent stimulation of adenylyl cyclase and epidermal growth factor receptor (EGFR). Also, methylation, acetylation, ubiquitination, and sumoylation can restrain ER action via multiple mechanisms (201).

The rapid effects of estrogen, such as increased cAMP concentration in minutes (without gene activation), suggest that ERs confined within the plasma membrane are accountable for this action (202, 203). Some studies indicated that post-translational modifications such as palmitoylation increase the receptor's association with the plasma membrane. In the plasma membrane, E2 drives cell survival through ERα non-genomic signaling and cell death through ERβ non-genomic signaling (204). Additional to the plasma membrane, ERs have been reported in organelles such as mitochondria (205) and the endoplasmic reticulum (149, 206). However, ERs non-genomic activity is not as well understood as the genomic pathways, and additional research is required in this area.

1.4.8 Enhancers and epigenetic mechanisms

ERs primarily control gene expression by binding directly to sequence-specific elements in DNA. Only 3% of ER binding sites are in proximal promoter area (within 5 kb) of the targeted gene, while the rest are present at distal regulatory components. These are named enhancers (181, 182) and can regulate transcription processes over extensive distances of countless thousand base pairs and from a location downstream or upstream of the location of transcription initiation (207). Enhancer activity models have elucidated the looping of chromatin. An enhancer far away in the distance from its target promoter on a linear chromosome can be brought into proximity in 3D space (208).

Some studies established ER functions via chromatin looping that brings transcriptional regulatory complexes of enhancers with the specific target promoters, connecting the ERs activity at distal locations to transcriptional start sites (TSSs) (209, 210). Chromosome conformation capture (HiC) detects chromatin interactions and, with the use of deep sequencing, maps these interactions in the 3D organization of the genome. This recent advancement has revealed that most of the enhancer-promoter interactions happen within large organizing components (between hundreds of kilobases) of the genome designated as topologically associating domains (TADs) (211). The utilization of the same technique shows that E2-ERα prompts global chromatin restructuring in MCF7 cells (212) and reorganizes open and highly transcribed chromatins, permitting genes to share the transcriptional complex and regulatory elements as reviewed in (201).

Genome-wide studies also suggested that many enhancers and promoters contain a typical histone mark. Histones flanking active promoters are marked by H3K4me3, while enhancers are marked by H3K4me1, with both marked by H3K27ac after activation (213, 214). Moreover, within the locality of highly expressed genes, multiple transcription factors are recruited to initiate transcriptionally more active clusters of enhancers in relatively small sections of DNA. These are called super-enhancers which regulate cell type-specific and disease-related genes, including oncogenes (215, 216). Super-enhancers can be categorized as coregulated loci, by computationally detecting peaks containing high transcription factor binding levels and intersecting with dynamic chromatin marks, such as H3K27ac and DNase I hypersensitivity mark (215, 217).

Enhancer RNAs are also hallmarks of active enhancers and are involved in enhancer activation and functional activity (218, 219). These are non-coding RNAs that are transcribed at enhancers as a result of RNA polymerase II action. A fascinating likelihood is that enhancer RNAs might partake in a structural task in developing or preserving enhancer-promoter loops. Indeed, new data supports this theory (220, 221).

1.5 ERβ role in CRC

1.5.1 Major challenges in the field

It has been 25 years since ERβ was discovered; however, ERβ expression and distribution in tissues and cells is unclear and still debatable. ERβ expression and its role in MCF7 (breast) and LNCaP (prostate) cancer have been extensively studied, in contradiction with clinical parameters. Further, ERβ is not present at the mRNA level in most cell lines. Fifteen out of 934 cell lines show ERβ expression based on RNA-Seq data (222). Only B-cell lymphoma and ovarian granulosa cell lines expressed ERβ. Although ERβ antibodies detecting protein expression, irrespective of mRNA raises the question of antibody specificity. Nonspecific antibodies are indeed a major problem in research (223, 224).

Our group made efforts to study and validate extensively used ERβ antibodies. We reported that the most widely used ERβ antibodies (14C8, PPG5/10) are not specific to ERβ protein. We have identified that only one of 13 antibodies (PPZ0506) specifically targets ERβ, and this antibody was not widely used. Using this validated antibody (PPZ0506), we have detected ERβ expression in human testis, ovary, granulosa cell tumors, and colon (225, 226). During the same period, Nelson and colleagues also reported the use of unspecific ERβ antibodies in research (227). A comparative study between WB and IHC has shown that antibody performance depends on the application and suggested validating antibody for the intended application.

The use of unspecific antibodies in literature raises the question of functional significance of ERβ, and caution must be exercised in interpreting data obtained from such experiments. Therefore, unspecific antibody-based data was excluded from the literature review, and only results also supported by mRNA evidence were considered.

1.5.2 Epidemiological evidence

It is a well-established fact that sex plays a vital role in CRC progression and survival. Men have increased progression and decreased survival compared to women of all ages (228, 229). Men with inflammatory bowel disease are at a 60% higher risk of developing colon cancer than women (20). Women benefit from significantly longer survival after rectal cancer resection than men. These results indicate that sex steroids may protect women from colorectal cancers (230, 231). Though not initially designed to explore CRC risk, the Women Health Initiative study established that use of hormone replacement therapy (HRT) protects women from CRC by reducing the risk up to 30% (232, 233). HRT is a combination of estrogen and progestin, and several studies have shown that estrogen alone contributes to large protective effects (234, 235). Also, the use of oral contraception has been shown to reduce CRC incidence by

approximately 14% (236). Consequently, oophorectomy (the removal of ovaries) in women increased CRC risk by 30% (237). These findings suggest that estrogen may protect against colon cancer, but the mechanisms by which this occurs remain unclear.

A meta-analysis study showed that soy consumption, which is rich in phytoestrogens, is associated with a 21% reduction of CRC risk in females but not in men, concluding that soy supplementation is synergistic with estrogen *in vivo* (238). Additionally, when individuals travel from an Asian nation to the United States, the occurrence of CRC in the immigrants increases to the average levels seen in the United States, indicating lifestyle and environmental factors role in etiology (239). Amongst the aspects related to the so-called *western* way of life, obesity occupies an important risk element regarding CRC. Notably, a positive relationship between the BMI and the frequency of colonic adenoma and advanced polyps was revealed in moderately young persons of both sexes and in premenopausal women with estrogen effects (240).

1.5.3 CRC suppression by ERβ

While ERα is upregulated in several cancers, most notably in breast cancer, where it promotes cell proliferation, ERβ expression appears to decrease during cancer progression. At the mRNA level, ERβ is lost in CRC while expressed in non-tumor colon tissue. ERβ has been identified as the predominant ER type in colon in many studies, while ERα protein is not detected in the colonic epithelium or CRC (241).

In ERα-knockout mice using the AOM tumorigenesis model**,** soy protein was shown to protect ERα-knockout mice and WT mice from colon cancer (242). However, studies in ovariectomized $Apc^{Min/+}$ mice treated with coumestrol and genistein showed that coumestrol lowered tumor number in the colon and small intestine while genistein was found to have no significant effect (243). However, estrogen has been shown to mediate protective effects in wild-type mice, but not in ERβ-knockout mice, supporting ERβ as a mediator of these effects (244). Therefore, the protective effects of CRC from E2 could depend on colonic ERβ (245).

Saleiro et al. demonstrated that in females, whole-body deletion of ERβ causes an increase in colon adenomas and size in the AOM-DSS mouse model. Besides, they found that the loss of ERβ increased the levels of β-catenin and inflammation related molecules (246). However, using the same model, a study by Heiman et al. found that ovariectomy protected female mice from colitis-associated tumor development and estradiol and medroxyprogesterone acetate (MPA) or both promoted tumorigenesis. Using ER mutant mice, they concluded that estrogen depends on ERα and ERβ for protumorigenic effects (247).

A recent study using different colitis models induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) showed that E2 reduced the expression of pro-inflammatory cytokines. Interestingly, the authors also noted increased proliferation and reduced apoptosis in ERβKO mice and suggested the differential protective effects of E2 in presence and absence of ERβ (248). Interestingly, ERβKO mice exhibited increased cellular proliferation of colonic crypt cells in

both colitis models (DSS and TNBS), indicating the importance of ERβ in the protection against colorectal tumors (246, 248). Similarly, enhanced intestinal tumorigenesis was demonstrated in ERβ knockout ovariectomized $Apc^{Min/+}$ mice (249).

Along with those above epidemiological and animal studies about estrogen and ERβ protective effects, there is significant evidence supporting tumor suppressive activity of ERβ in cell line models. Re-introduction of ERβ in human colon cancer cell lines (SW480, HT29 and HCT116) downregulates IL6 inflammatory marker and reduces the expression of numerous oncogenes, for example, MYC, MYB and PROX1 (250, 251). Strikingly, ERβ has been shown to increase p53 signaling and reduce β-catenin levels, which increased apoptosis and reduced cell proliferation in LoVo colon cancer cells (252). These studies combined strongly support a role for intestinal ERβ as a target for chemo-preventive therapy in colon cancers, however, the molecular mechanisms behind ERβ function are not well understood.

1.5.4 ER cross-talk

To coordinately regulate target genes, ER's recruit several transcription factors as partners to activate or repress hundreds of target genes at once (181, 190, 253). Many studies have shown a potential cross-talk between ERα and other transcription factors; only a few studies were conducted on ERβ cross-talk. Although ERβ shares several transcriptional mechanisms with ERα, there are unique ERβ mechanisms. ERβ undergoes conformational changes specific to each ERE, which result in the recruitment of differential coactivators (254). While both ERs have almost identical DBDs, ERβ has a lower affinity to ERE half-sites than ERα (255, 256). These differences between half-site binding and ERE-dependent conformational changes could contribute to the differences between ERα and ERβ in transcription activity (257).

Previous studies have identified many pioneering factors and coregulators crucial for transmitting the hormonal signal to the transcriptional machinery. FOXA1 is identified as a pioneer factor that binds to condensed chromatin allowing the recruitment of other transcription factors to the DNA (258). Particularly, FOXA1 reprograms ERα recruitment at *cis*-regulatory components in breast cancer cells (182). Numerous sequencing initiatives, along with further research, identified regulators of ERα (FOXO3A, FOXM1, GATA3, ERBF-1) (259), as well as ERβ (CREB in endometrial cells (260), AP2α in prostate cancer cells (261), and Clock/*Bmal1* in mouse models (262). Recently a couple of studies reported that tet methylcytosine dioxygenase 2 gets involved in ER complex along with GATA3 and acts as a necessary component for ER binding to chromatin (263, 264).

Several studies reported that different steroid receptors and transcription factors modulated p65 chromatin binding. In the breast cancer field, numerous studies indicated that ERα represses p65 to exert anti-inflammatory activity (265-268), whereas some studies have shown that ERα and p65 work together to increase gene transcription synergistically (189, 269-274). It is, therefore, unclear whether ERα and p65 activate or repress each other, but an interaction between ERα and p65 has been consistently reported. In a study, Franco et al. (275) revealed that TNFα-activated NFκB reprograms ERα to a subcategory of NFκB-binding sites, resulting

in increased production of enhancer RNA transcripts, an activity linked beforehand to enhancer activation by ER (276).

The interaction between ERα and NFκB is shown at the baculoviral inhibitor of apoptosis 856repeat-containing 3 (BIRC3) target gene. TNFα activated NFκB binds to the BIRC3 promoter and prepares it for ERα binding, which in return enhances TNFα-induced BIRC3 activation (277). Besides, TNFα intensely transforms the ERα enhancer setting in an NFκB-dependent fashion (275, 278). Our group has reported that also ERβ regulates NFκB target genes, including downregulation of Il-6, which is the main target of the NFκB pathway (279). However, there is no data available for ERβ coactivators in colon cells, thus understanding the cross-talk between ERβ and its co-activators is essential for understanding its function. Based on that, in this thesis, I am exploring whether ERβ and NFκB interact specifically in colon cancer. Learning more about NFκB signaling and how it is influenced by estrogen signaling should lead to better therapeutic approaches in the future to target it in specific cancer types.

1.5.5 ER cistrome studies

Albeit ERα inhibitors have been utilized in clinics since the 1980s, the mechanism of ERα's genomic activity on a genome-wide scale remained mysterious for a long time (280). Primary progress of chromatin immunoprecipitation (ChIP) together with DNA microarray analysis (ChIP-on-chip) techniques, ERα genomic binding, was identified on a genome-wide scale for the first time (182). Through the expansion of high-throughput sequencing methods, several ERα ChIP-Seq studies were executed in cell lines and some in tissues (281). Almost 95% of the entire ERα-binding locations are premised at distal *cis*-regulatory components (henceforth labeled as 'cistromics') that later acquired recognition as enhancer areas (280, 282).

Several studies have been published on ERβ ChIP-Seq in breast mainly using exogenous expression of ERβ. In MCF7 cells, studies using different antibodies showed between ~400 to 38 000 ERβ binding sites (235, 283-285). Several studies in different cell lines (286) or tissues (287, 288) identified ERβ binding sites somewhere between 5000 to 14 700 binding sites; few studies used antibodies not highly specific to ERβ. Though differing studies indicate changeable statistics of ER binding actions (even in the same cell line), possible because numerous enhancers might control a single gene (289), or certain variances are attributable to the experimental conditions and the lack of specificity of antibodies that were utilized.

2 RESEARCH AIMS

General aim

In epidemiological and animal studies, estrogen receptor beta (ERβ) has been shown to protect from colorectal cancer (CRC), but the mechanisms are poorly understood. Thus, the overall aim of this thesis is to elucidate the role of ERβ and evaluate its potential as a therapeutic target in prevention of colorectal cancer. The research articles presented in this thesis identify molecular mechanisms of ERβ involved in preventing CRC formation and development and propose novel insights involved in CRC prevention.

Specific aims

Paper I: To test if intestinal epithelial ERβ mediates protective role in CRC, using a colitis-induced tumor mouse model with tissue specific knockout of ERβ and to investigate any sex differences.

Paper II: Determine the genome-wide binding pattern and regulation of ERβ in colon cancer cells and identify its cofactors.

Paper III: Elucidate the impact of estrogen signaling in HFD induced colon inflammation in both sexes.

Paper IV: Evaluate the cross-talk between ERβ and p65/TNFα inflammatory signaling in colon cancer cells.

3 MATERIALS AND METHODS

3.1 Mouse models

Fundamentally, animal models of human disease are valuable models for studying several diseases, including the development of cancer. Animal models also play a crucial role in translating scientific studies into human disease and in predicting the future of novel therapeutic drug targets. The animal studies got approved by the Swedish Regional Board of Animal Research. In Sweden, the use of laboratory animals requires well-controlled ethical permission, controlled premises, and adherence to high animal welfare requirements. In the use of animals, the "3Rs" recommendations that encompass Replacement, Reduction, and Refinement were adopted in 1959 (290). Replacement: Animal experimentation is avoided wherever possible, and *in vitro* models should be used instead. Reduction: use the smallest number of animals required for statistical power. Refinement: reduce animal suffering and improve animal welfare.

The mice were constantly provided with a regular chow diet and water and were kept on a 14-hour light, 10-hour dark cycle. Primarily, in the paper I, CA-CRC was induced in WT mice and mice that specifically lack ERβ in intestinal epithelial cells (referred to as ERβKO^{Vil}). Regarding paper III, C57BL/6J mice with HFD-induced obesity were used.

3.1.1 ERβKOVil mouse

ERβKOVil mouse model maintained on C57BL/6J background strain was generated by Cre/loxP recombination technology. ERβflox/flox mice with ERβ exon 3 (B6.129X1-Esr2^{tm1Gust}) flanked by two loxP sites were bred with transgenic mice expressing Cre recombinase under the control of the enterocyte-specific Villin promoter. Cre is therefore only expressed in the intestine, where it deletes the DNA sequence between the loxP sites in a site-specific manner. ERβ gene exon 3 generates the first zinc finger in the DBD. Its knockout led to frameshift in the coding region following splicing from exon 2 to exon 4, resulting in a frameshift and an early stop codon, reduced mRNA levels (due to nonsense-mediated decay), and no ERβ protein expression. ERβflox/flox mice without Cre allele were used as controls (denoted as WT). The opportunity to characterize the function of ERβ specifically in the intestinal epithelium is a benefit of ERβKOVil mice. It is also worth noting that ERβ is lost in the small intestine. The full-body knockout of ERβ in mice results in phenotypes with fertility issues with few littermates (291), but so far, no major effects were observed in the colon of ERβ knockout mice in standard conditions. However, challenging ERβ knockout mice with inflammatory conditions or obesity mice gives more pronounced effects due to lack of ERβ.

3.1.2 AOM/DSS-induced colitis

Azoxymethane (AOM)/dextran sodium sulfate (DSS) model has been used extensively in research to mimic human CA-CRC features. The AOM/DSS induces distally located invasive tumors and molecular modifications in β-catenin, pro-inflammatory cytokines (TNFα), similar to human CA-CRC (292-294). ERβKOVil and WT mice between 5 and 10-weeks were

intraperitoneally injected with colonic carcinogen AOM (10mg/kg) on day 1 and then accompanied by colonic irritant DSS treatment in drinking water starting from day 7 to 14. The DSS cycle was then repeated three times, with two weeks of regular drinking water in between. For the duration of the DSS treatment, the mice lose weight, have diarrhea, and develop rectal bleeding, and all these different parameters were monitored using a score sheet. The mice recover during the phase of drinking normal water and, in due course, acquire colitis followed by adenomas. Following 9 or 15-16 weeks of AOM injection, the mice were sacrificed. Notably, the AOM/DSS technique has excellent reproducibility and potency, besides being a simple and inexpensive method of application. It is worth noting that different strains of mice respond differently to this treatment and that their microbiota composition can influence the efficacy of tumor development.

3.1.3 High-fat diet (HFD) induced colon inflammation and obesity

Male and female C57BL/6J mice bred in-house at the animal facility and aged 5 to 6 weeks were fed an HFD (60% kcal fat) or a control diet (CD) (10% kcal fat) for 13 consecutive weeks. The mice were treated with vehicle or estrogenic ligands, E2 (0.05 or 0.5 mg/kg), PPT (2.5 mg/kg), and DPN (5 mg/kg) for a total of 9 injections for 3 weeks before sacrifice. The ligands were prepared in a mixture containing 40% PEG400, 5% DMSO, and 55% water. In comparison to the regular western diet, which has approximately 45% calories from fat, this diet has a higher fat content. The physical properties of the HFD diet, which easily breaks into powder, creates a problematic situation regarding monitoring the food intake. Measuring their exact caloric intake, and controlling their actual consumption, is therefore, a considerable challenge *(295)*.

3.1.4 Tissue collection

Weighed colons were cleaned and opened along the axis, and the number and size of adenomas were recorded. The colons were fixed with 4% formaldehyde for 24 hours and kept in 70% ethanol before embedded in paraffin for immunohistochemistry (IHC) or the fresh tissue is directly frozen in liquid nitrogen for analysis, including with RNA extraction and quantitative reverse-transcription PCR (qPCR). Also, fresh tissues were directly embedded in OCT and frozen on dry ice and stored at 80°C. A pathologist who was unaware of the mouse genotype scored the colitis in hematoxylin and eosin (H&E) stained sections. The colitis score (0-12) was calculated: the severity, the extent of hyperplasia, level of ulceration, and the size of the affected areas were all graded between 0-3.

3.2 *In vitro* studies

3.2.1 Colon cancer cell lines

Well-characterized cell lines enable reproducibility and resolve most of the ethical issues that come with using animal and human tissues. Due to these reasons, cell line models continue to be the most widely used in research worldwide. The foremost objective in choosing CRC cell lines HT29 and SW480 in Paper II and IV was to create an optimized, well-controlled system

for studying the genome-wide binding of ERβ. HT29 is an epithelial-like human colorectal adenocarcinoma cell line generated from a 44-year-old female colon adenocarcinoma tumor (296). The SW480 was obtained from a 50-year-old male with type B colorectal adenocarcinoma (297). Differences include that HT29 cells are CIMP (CpG island methylator phenotype) positive, whereas SW480 cells are CIMP negative. The HT29 contains WT KRAS, PTEN genes, and mutations in BRAF (V600E), PIK3CA (P449T), and TP53 (R273H). SW480, on the other hand, has WT BRAF, PIK3CA, and PTEN and mutations in KRAS (G12V) and of both TP53 alleles (R273H and P309S) (298). The P53 allele in the SW480 cell line is a double mutant, while the P53 allele in the HT29 cell line is a single mutant, which may result in a different chromatin complex with P65 (298). We also used ERα-positive MCF7 breast cancer cells with tetracycline-induced expression of ERβ in paper II to compare tissue-specific ERβ chromatin-binding between colon and breast. In Paper IV, we used MCF7 parental cells to compare p65 cistrome between colon and breast. Due to the lack of ERβ in CRC cells, ERβ has been exogenously added using a lentivirus system, which has been generated and characterized previously (250, 251, 299).

Cell lines were cultured in a standard cell culture medium comprising 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were serum-starved using 1% charcoal-stripped FBS and DMEM-phenol red-free media. Regular FBS contains hormones, such as estrogen and phenol (which have weak estrogenic properties). *In vitro* models are an excellent choice to dissect molecular mechanisms; however, they have a drawback when studying a complex disease like CRC because of the lack of tissue microenvironment that influences tumor growth and metastasis *in vivo*.

3.2.2 Chromatin immunoprecipitation

In many major cellular processes, such as transcription, splicing, replication, and DNA repair, DNA-binding proteins play crucial roles. These proteins include transcription factors that preferentially bind to specific sequences of DNA and histone proteins that form the nucleosome core, which is the chromatin's basic unit. To study genome-wide binding of transcription factors, chromatin immunoprecipitation (ChIP) combined with microarrays (ChIP-on-chip) or sequencing (ChIP-Seq) has become standard techniques (300, 301). In brief, the cells were cross-linked to capture protein-DNA interactions, and the chromatin was sheared into small fragments in the range of 200-600 bp by sonication. DNA-protein complexes were immunoprecipitated with an antibody specific to the protein of interest. Cross-links were reversed, and the released DNA was sequenced to determine the sequences bound by the protein (301, 302). ChIP-Seq performed with a specific antibody provides an accurate map of binding sites for transcription factors and other DNA-binding proteins, and this information is crucial for understanding biological processes. Integrating ChIP-Seq data with functional genomic and transcriptomic data (RNA-Seq) can provide a global view of chromatin accessibility and regulation of gene expression (303). ChIP-Seq is the primary technique used in papers II and IV, where it is accompanied by RNA-Seq or microarray data.

It is important to remember that each method has its limitations. From a technical perspective, for a high-quality ChIP experiment, a highly specific antibody and a large number of cells (~10 million) are usually required (304, 305). In addition to direct binding events, the signal from ChIP-Seq also includes indirect binding in which one factor interacts with another DNA-binding factor (tethering). It is essential to distinguish between direct DNA binding and tethering events, but this is not directly achieved from ChIP-Seq data but requires further analysis, in silico or experimental (301). Recent technological advances in the ChIP-Seq protocol have made it possible to test samples with limited cells, increase the accuracy of the genomic location of binding events, and test multiple binding events (306-309).

The ChIP-Seq data pipeline addresses various aspects of the assessment and management of data: the identification of artifacts, filtering low-quality reads, estimating library complexity, sophisticated reading alignment and peak calling algorithms, and downstream analytical tooling (300). For a ChIP-Seq comparison, a common normalization approach uses the total number of mapped reads per million to adjust for biases generated by sequencing depth between samples. This approach works only to correct technical artifacts but cannot correct biological differences like different peak numbers. Several publications have pointed out these biases (310-313) and indicated the need for an initial normalization step. Various strategies have been employed, such as relative level difference and absolute level difference using synthetic histone spike in normalization (314, 315). Recently, another normalization strategy called parallel factor ChIP addressed these challenges by using a second antibody against the target chromatin as an internal control (316). Although the need for an exogenous spike-in is eliminated in this technique, its dependence on antibodies might cause reproducibility concerns. There are no gold standards available for ChIP-Seq data normalization and analysis. Hence the optimal method should be selected based on prior system knowledge and sequenced sample statistics (300, 302). There are web-based services such as ChIPseek (317), Nebula (318), and CSA (319) that are available to analyze ChIP-Seq data.

The first method for collecting single-cell chromatin data has been published using the "Drop - ChIP" method (320). This method uses a droplet microfluid labeling system for individual cell chromatin before immunoprecipitation and reports ~800 reads per cell. Recent development using the microfluidic droplet method has a higher resolution with ~10,000 reads per cell. Single-cell ChIP-Seq using this method was performed in breast cancer and provided a new alternative to study complex chromatin heterogeneity in tumors (321).

3.2.3 Luciferase assays

We performed luciferase assays to determine how ERβ, AP1, or NFκB can affect transcription and potentially cross-talk. The reporter gene expression is kept under the control of firefly or renilla luciferase. We used luciferase constructs with estrogen response element (ERE), NFκB response element (NFκB-RE) (Paper I), or TPA response element (TRE) (Paper II) in their

promotors. Plasmid with renilla luciferase reporter was used as a control to normalize enzyme activity of firefly luciferase.

Firefly and renilla luciferase are two bioluminescent forms of proteins that emit light in response with their substrate D-luciferin (firefly) or coelenterazine (renilla), and the produced light intensity is proportional to the protein concentration in the sample. The reporter gene's expression is directly proportional to the protein expression allowing for monitoring of small changes in expression.

3.2.4 Statistical analysis

For statistical analysis, GraphPad Prism was used (GraphPad Software Inc). The data is provided as a mean ± standard error of the mean (SEM). For comparison between two groups, a two-tailed Welch's t-test or paired t-test (if paired data) was used. For comparisons between multiple groups, one-way and two-way analysis of variance (ANOVA) were used, followed by Fisher's LSD test for *in vivo* data or Tukey's multiple comparisons test.

4 RESULTS

4.1 Paper I: Intestinal estrogen receptor beta suppresses colon inflammation and tumorigenesis in both sexes

Studies in full-body ERβ knockout mice and selective activation of ERβ with ligands have previously confirmed the notion that estrogen via ERβ protects aginst CRC (244, 322-325). Our group's research with exogenously expressed ERβ has shown the antiproliferative and antitumorigenic effects of ERβ in both colon cancer cell lines (326-329) and xenografts (327). Thus, selective activation of ERβ is a potential choice for CRC preventative therapy, but its mechanism of action needs to be elucidated. ERβ has been detected in a few immune-related organs and some immune cells (225), and it is possible that ERβ could impact CRC development through the immune system. The previous studies which explored the role of ERβ in CRC using full knockout animals could not exclude possible systemic ERβ effects via the immune system. Also, with one exception (324), all studies were conducted only in female mice (244, 325). However, ERβ is expressed in colon and rectum in both men and women, men also synthesize estrogen (primarily E1), and ERβ can be activated by dietary phytoestrogens. Thus, men are also expected to have some ERβ activity. Hence, we here attempt to understand the physiological relevancy of ERβ in both sexes using colitis-induced CRC (CA-CRC) in male and female mice that lack ERβ, specifically in intestinal epithelium ($ERβKO^{Vil}$). We have used the AOM/DSS model to induce CA-CRC, which reflects the histopathological and molecular environment of human CA-CRC.

Using RNAscope and IHC with a validated antibody (225), we confirmed ERβ expression in normal colon and rectum epithelia of both human and mouse, and lack of expression in CRC, which supports our underlying hypothesis that ERβ can protect against CRC formation. We observed that loss of intestinal ERβ increases the number of tumors in males and the tumor size in females (Figure 6). One interesting finding is that loss of ERβ increased the pro-inflammatory cytokine *Tnfα* expression in both sexes, most prominently in males. Moreover, we noted that several TNFα and NFκB target genes, such as *Il6* and *Il1b,* strongly increased upon loss of ERβ, following AOM-DSS treatment, especially in males. On the other hand, in females, lack of epithelial ERβ increased both ulcerative areas and reduced ulcer healing. We have thus demonstrated that both sexes are protected by intestinal ERβ from CRC in a sex-dependent manner. The tumor size and incidence differences between males and females may be partly due to these specificities or microenvironmental signals, and differences in their immune system, resulting in different colon epithelial gene expressions between males and females.

We have also previously shown that the microbiota diversity in these mice appeared modulated by ERβ (330). To circumvent possible impact by microbiota effects and explore if ERβ could protect the epithelial cells from TNFα-induced damage, we treated intestinal organoid cultures generated from WT and $ERβKO^{Vil}$ mice of both sexes, with TNFα and a selective agonist for ERβ (DPN). The *ex vivo* crypt formation, which quantifies regenerative growth, was decreased by TNFα treatment in both sexes of WT and $ERβKO^{Vil}$ mice. In both sexes of WT mice, ERβ-

selective agonist DPN significantly counteracted the TNFα-induced epithelial cell damage but did not do so in ERβKO^{Vil} mice. This data demonstrates that the activation of intestinal ERβ protects both sexes against TNFα-induced epithelial cell damage.

Further, we explored these molecular mechanisms in two human colon cancer cell lines HT29 and SW480, using transcriptomic and genome-wide studies. We found that ERβ modulated a proportion of the TNFα-regulated transcriptome and inhibited most of these genes in CRC cells. Next, we tested whether ERβ can impact the translocation of NFκB subunit p65 or transactivation of TNFα. We observed that ERβ affected neither the translocation of p65 nor the transactivation of TNFα in either of the cell lines. We could confirm that ERβ modifies the expression of NFκB regulated genes also in mice, such as *Atf3, Bcl3,* and reduces pro-inflammatory signaling by lowering levels of chemoattractants *Ccl2* and *Ccl4*. NFκB targets ATF3, BCL3, and BIRC3 also had ERE elements in close proximity, indicating that ERβ has the potential to bind these targets. Using ChIP-qPCR, we have confirmed that ERβ binds to NFκB targets, ATF3, BCL3, and BIRC3 in the promoter or enhancer regions, indicating biological cross-talk between ERβ and p65. Further, using the ERE-luciferase transactivation assay in cell lines, we found that TNFα could enhance the transactivation of ERβ. This suggests that an inflammatory condition increases ERβ activity, which successively inhibits TNFα-mediated signaling and thus reduces the inflammation, implying an inhibitory feedback loop.

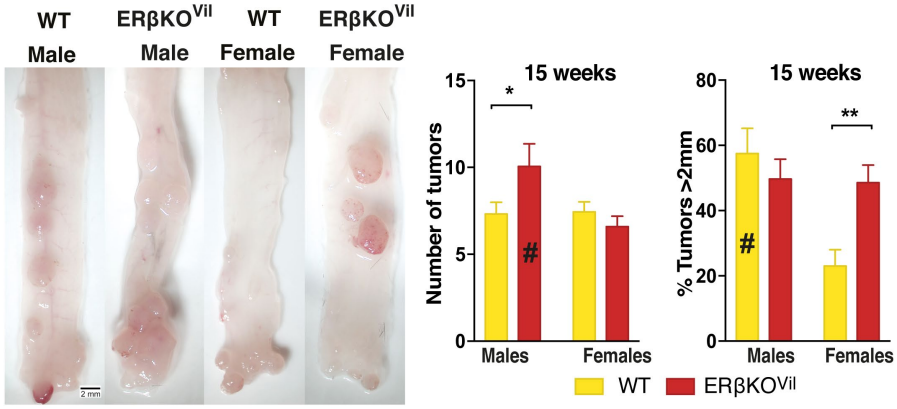

Figure 6. (A, B) Intestinal ERβ protects both sexes from colitis-induced CRC in a sex-dependent manner. Loss of intestinal ERβ increases the number of tumors in males while increasing the tumor size in females (226).

In this study, we tested only a limited number of promoter regions. Further performing ERβ and p65 ChIP-Seq is significantly more informative and reveals the molecular basis for this cross-talk, which is addressed in paper II and IV. In conclusion, our data indicate that ERβ, expressed specifically in the intestinal epithelia, protects from colitis-induced CRC in a sex-dependent manner by attenuating TNFα- NFκB pathway.

4.2 Paper II: Genome-wide estrogen receptor β chromatin binding in human colon cancer cells reveals its tumor suppressor activity

The antitumorigenic effects of ERβ have been previously reported by our group in different CRC cell line models, where re-expression of ERβ reduced cell proliferation, migration, and invasion (245, 250, 251, 299). In paper I, the deletion of intestinal ERβ in mice enhanced tumorigenesis, indicating the importance of colonic ERβ in protecting from tumors. All these studies strongly support a role for intestinal ERβ as a target for chemopreventive therapy in colon cancers; however, the ERβ mechanisms of action at the chromatin level in colon cells have not been studied yet. So far, only a few studies have mapped ERβ binding sites, mostly in MCF7 breast cancer cells (16, 17). The ERβ chromatin binding in the colon is unknown.

To determine colonic tumor protective effects of ERβ through its activity as a transcription factor, we aimed to characterize its genome-wide chromatin binding sites and identify its target genes in colon. One of the challenges in studying ERβ is its low native expression in tissues and its lack of expression in immortalized cancer cell lines (257). Introducing ERβ into cell lines is a way of restoring its loss of endogenous expression to study its impacts. For this purpose, we performed ChIP-Seq on previously characterized CRC cell lines with transduced ERβ expression, HT29-ERβ, and SW480-ERβ (299, 327). The quality of a ChIP-Seq study is highly dependent on the specificity and selectivity of the antibody used, and we further optimized and evaluated the highly validated ERβ antibody PPZO506 for application in ChIP-Seq (225).

We mapped 2977 and 1853 ERβ binding sites in HT29, SW480 cells, respectively. In line with previous ER cistrome studies, most ERβ binding sites were located within intronic (43%) and intergenic (40%) regions. We found that the majority of binding sites contain canonical ERE motif, indicating that ERβ binds directly to DNA with its DNA-binding domain. Further, a relatively large proportion exhibited AP-1 motif, indicating its known interaction with the AP-1 complex. We also discovered motifs (TCF, ELF3, and KLF5) that have not previously been associated with ERβ, indicating potential novel cross-talk (Figure 7). Overall, the most enriched biological functions among genes closest to ERβ binding sites include cell migration, cell-cell adhesion, and transcriptional regulation in both cell lines. These are processes that ERβ is known to affect.

To determine whether ERβ binding is functionally relevant in transcriptional regulation of genes, we linked ERβ ChIP-Seq data with corresponding gene expression data from SW480 and HT29 cells. Associated with ERβ binding sites, ERβ regulated genes are mainly involved in proliferative Wnt signaling, NFκB signaling, and insulin-like growth factor signaling pathways. We identified that ERβ strongly upregulated CST5, a candidate tumor suppressor in colon cancer, and downregulated the LRP6 oncogene, which promotes aberrant β-catenin signaling. To transcriptionally regulate the oncogenic Wnt pathway, β-catenin depends on TCF family proteins, as it lacks a DNA-binding domain (331). A significant fraction of ERβ-binding sites contained TCF4 motif in both SW480 (12%) and HT29 (19%) cells.

To investigate whether ERβ impacts the epigenome, we studied the histone acetylation mark H3K27ac which represents transcriptionally active enhancers, promoters, in presence and absence of ERβ. A significant fraction of H3K27ac binding sites were modulated by the presence of ERβ in both HT29 (4159 enhanced, 4835 reduced) and SW480 cells (3793 enhanced, 3596 reduced). Genes located by ERβ-modulated H3K27ac sites were also overrepresented among functions such as cell migration, apoptosis, and Wnt signaling.

To explore whether or to which extent ERβ binding is tissue-specific, we compared our ERβ cistrome data with that of breast cancer cell line MCF7, using the same antibody and method. The predominant ERβ-binding motifs in breast were ERE, AP-1, and GATA4. We found no enrichment for TCF and KLF motifs in breast, indicating that ERβ tethering with TCF and KLF5 is specific to colon cancer and thus cell context dependent. TCF and KLF5 chromatin accessibility was dysregulated in polyps associated with CRC invasion, according to a recent study that used ATAC-Seq to construct single-cell chromatin maps of polyps and CRC. TCF motifs were highly enriched in polyps, indicating increased chromatin accessibility, whereas KLF motifs chromatin accessibility was lost during cancer progression (332). KLF4 was also identified as a potential tumor suppressor in CRC (333). One could speculate that ERβ directly or indirectly competes with β-catenin to bind TCF proteins and controls the genes or regulatory elements involved in the early events of polyp transforming into CRC.

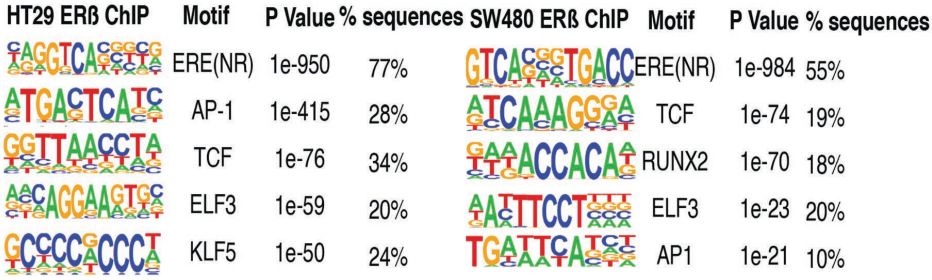

Figure 7. *De novo* motif analysis identified TCF and AP-1 motifs in ERβ binding sites in both colon cancer cell lines (334).

The use of exogenously expressed ERβ instead of native expression is one of the shortcomings of this study, and also that the cell lines are from colon cancer instead of non-tumor colon epithelial origin. However, the levels of ERβ in normal epithelial and non-tumor cell lines are too low to allow high-quality ChIP-Seq data, and non-tumor colon cell lines are difficult to culture and transduce. In conclusion, this study mapped genome-wide ERβ binding sites and identified the epigenetic changes by ERβ in colon cancer cell lines for the first time.

4.3 Paper III: High-fat diet and estrogen impact the colon and its transcriptome in a sex-dependent manner

Sex and lifestyle factors, like dietary factors, are important risk factors in CRC development. Intake of a High-fat diet (HFD) affects the colon first and modulates intestinal permeability, microbiota composition, and induces inflammation (81-83). A meta-analysis of 56 cohort and case-control studies found that obesity increases the risk of CRC, and a 5 kg/m^2 increase in body mass index (BMI) increases the risk of CRC development by 18%, and a BMI above 30 kg/m^2 increases the risk by 41% (79). Obesity impacts inflammation, gut microbiota, hormones such as estrogen, insulin, and leptin which all play a role in CRC (335, 336). Estrogen has shown to protect against obesity and metabolic syndromes, as reviewed in (337). In study I, we also found that ERβ protects from CRC in a sexually dimorphic manner, with more tumors in males and larger tumors in females. However, no studies have been conducted to investigate colonic sex differences in relation to obesity, metabolic dysregulation, and estrogenic ligands. In this study, we focused on determining if estrogen affects the colon microenvironment during HFD-induced inflammation and on dissecting sex differences. For 13 weeks, we fed mice an HFD with 60% fat or a control diet (CD) with 10% fat. In the last three weeks prior sacrifice, mice were treated with different estrogenic ligands. To investigate sex differences on the colon transcriptome, we performed RNA-Seq on male and female distal colon tissues.

The colon transcriptome of CD-fed mice revealed that a high proportion of genes (1564) were differentially expressed between males and females. Under HFD treatment, the sex difference was less prominent, with 364 differentially expressed genes. About a quarter of the transcriptomic sex differences found under HFD were also observed under CD. Among these stable sex difference genes, the most overrepresented genes belonged to the circadian rhythmic pathway and included key genes *Npas2* and *Bmal1*(*Arntl*), which were higher expressed in females compared to males. Further, separate comparisons showed that HFD modulated different genes in the sexes. Significantly overrepresented functions of genes regulated in males were cell cycle and hypoxia, while for females, it was lipid metabolism, steroid hormone, and Wnt signaling. Hypoxia can induce inflammation, and in IBD, hypoxia-inducible factors (HIF-1α and HIF-2α) are highly present (338). In paper I, we also found strong inflammatory signaling and significantly increased *Il6* and *Il1b* in WT males compared to WT females, suggesting sex differences. We also identified that HFD-induced sex differences in colon length (a shortened colon is indicative of inflammation), fasting glucose, and crypt proliferation. A shorter colon was found only in males but not in females. Further, HFD increased crypt proliferation and fasting glucose levels in males but not in females. On the contrary, HFD enhanced $F4/80^+$ macrophage infiltration in both sexes but more strongly in females.

To determine if ERβ could modulate inflammation, we treated mice with estradiol (E2, activates all ERs), a ligand selective for ERα (PPT), and a ligand selective for ERβ (DPN). Treatment with E2 opposed the HFD-induced gene expression, particularly on genes involved in metabolic processes and inflammation. These results clearly indicate that E2 opposes the HFD dysregulated transcriptome, possibly through ERs present in the colon or through improved metabolism in general (Figure 8A). E2 and PPT both controlled body weight gain and total fat, which impacts the overall metabolic profile, which is in accordance with previous studies indicating that E2 through ERα reduces food intake (339) and regulates the body fat distribution (340). The impact in the colon was mainly seen through E2 and DPN, which counteracted HFD-induced F4/80^{+} colon macrophage infiltration in both sexes and Ki67 proliferation in colon crypts of males. All three ligands E2, DPN, and PPT significantly restored the HFD reduced *Bmal1* gene in females. We further identified ERβ binding to BMAL1 and NPAS2 using ChIP-Seq data (from paper II) and correspondingly decreased gene expression of *Bmal1* and *Npas2* in the female colon with loss of intestinal ERβ (Figure 8B-D). This study concludes that HFD impacts the colon microenvironment with prominent sex differences. Estrogen modulates the HFD induced transcriptome and, through ERβ, counteracts HFD-dysregulated clock genes, macrophage infiltration, and cell proliferation in a sex-dependent manner.

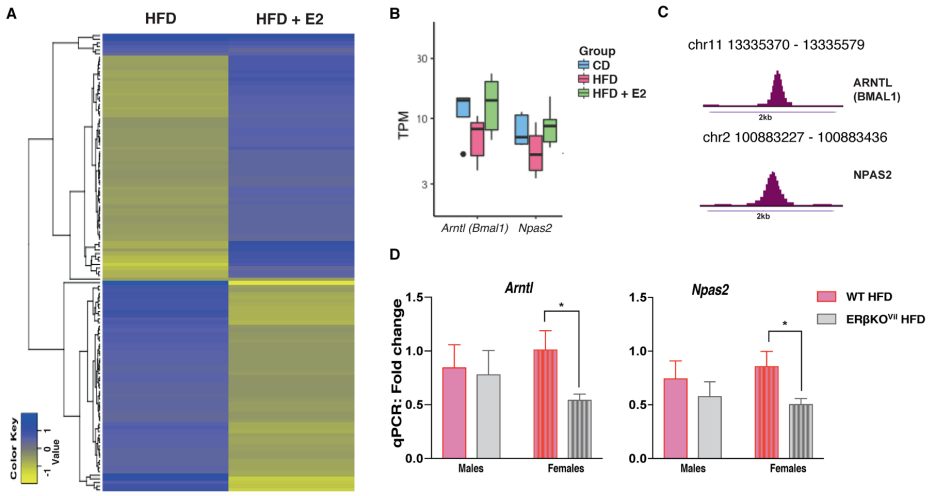

Figure 8. (A, B) E2 opposes the majority of HFD induced gene dysregulation in colon microenvironment, such as dysregulation of clock genes BMAL1 and NPAS2 in males (C). Interestingly, we identified ERβ binds to clock genes in colon cancer cells using ChIP-Seq (D) The clock genes were dysregulated specifically in the absence of intestinal ERβ upon HFD treatment, indicating direct regulation of core clock genes in females by ERβ (341).

4.4 Paper IV: Estrogen receptor beta influences the inflammatory p65 cistrome in colon cancer cells.

In paper I, we showed that ERβ protects against epithelial damage by repressing TNFα signaling *in vivo*. ERβ also binds in the vicinity of NFκB target genes and modulates the expression of TNFα regulated transcriptome in CRC cell lines. In paper II, we also find that ERβ chromatin-binding sites were enriched adjacent to genes involved in NFκB signaling. The ERs can interact with other transcription factors to promote or suppress transcription. In particular, a cross-talk between ERα and NFκB in breast cancer has been identified, and some studies (191, 192) support that ERα inhibits NFκB activity; on the contrary, other studies claim that ERα promotes NFκB activity (193, 194). The DNA binding domain is highly conserved between ERα and ERβ, but the two proteins differ significantly in their N-terminal domains and their ligand-binding domains (134, 136). Therefore, we hypothesized that ERβ transcriptionally modulates NFκB signaling by modulating its chromatin binding at the genome-wide level, for example, through protein-protein interaction or chromatin-binding competition. To experimentally demonstrate interactive cross-talk between p65 and ERβ, we performed p65 ChIP-Seq in CRC cells upon TNFα treatment in presence and absence of ERβ and compared with corresponding gene expression data.

We identified 3151 and 1459 p65 binding sites in HT29 and SW480 cells. They were located by genes involved in known biological functions such as TNFα signaling, cell migration, apoptosis, and more particularly circadian rhythm. The circadian rhythm has not been linked to p65 before, and this novel potential role in the colon paves the way for new studies. To compare p65 cistrome between different tissues, we have used publicly available p65 ChIP-Seq data from MCF7 cells (also performed using double crosslinking). Comparison of p65 chromatin-binding sites revealed that to a large extent, they are different between the breast and colon cancer cell lines, indicating that NFκB works uniquely to control transcriptional regulation in different cancers. The two p65 ChIP-Seq studies used different antibodies and protocols, which might also impact this comparison.

Further, we identified that ERβ modulates p65 chromatin binding in both CRC cells but in a cell line-specific manner. In paper III, we showed that ERβ restores the core clock genes dysregulated by HFD. Here, in paper IV, we found that ERβ modulates the p65 binding to core circadian genes CLOCK and BMAL2, further strengthening the intrinsic cross-talk between ERβ, clock genes, and p65. In HT29 cells, ERβ generally reduced p65 chromatin binding while it increased p65 binding in SW480 cells. However, in terms of gene expression, ERβ reduced p65 transcriptional activity in both cell lines. It is possible that biological, genetic, or epigenetic changes between HT29 and SW480 cell lines impact this cross-talk. Some notable differences are that the SW480 cell line contains double mutant p53 alleles (298), whereas HT29 has a single p53 mutation, which might result in different chromatin complexes with p65. Previous studies showed p53 and p65 interactions in various cancers with different outcomes (342-344), and it was shown in SW480 cells the double mutated p53 variants were able to impact p65

binding (345). Also, HT29 is a female cell line, while SW480 has a male background. Our results should be verified with more cell lines to further understand this cross-talk.

Some methodological and potential shortcomings of this study include that cells were treated with different TNFα timings and concentrations for the Chip-Seq study compared with the gene expression experiments. Genomic binding occurs immediately after p65 translocation, and we have therefore used 30 minutes TNFα treatment for ChIP-Seq experiments. The regulation of gene expression by p65 is a slightly later event as it occurs after chromatin binding and the mRNA remains in the cell for a longer time; hence we used a 2-hour treatment for gene expression analysis. In paper II, we demonstrated that ERβ chromatin binding occurs independently of ligand in the CRC cell lines. ERβ has previously been shown to have ligand-independent functions in cell lines, which may be a consequence of overexpression or that the high level of growth factors activates ERβ through phosphorylation. Hence, we did not treat the cells with E2 to activate ERβ. Furthermore, the addition of TNFα to estrogen (E2) treatment in the MCF-7 breast cancer cells has been reported to alter the ERα enhancer landscape in an NFκB-dependent manner (275). In future studies, it would be interesting to investigate whether the ERβ cistrome is affected by TNFα treatment. Here, we defined how ERβ modulated p65 chromatin binding in colon cancer cells and provided the mechanistic basis for understanding the p65 cistrome and corresponding TNFα-induced response; further revealing a cross-talk between ERβ and the NFκB pathway (Figure 9).

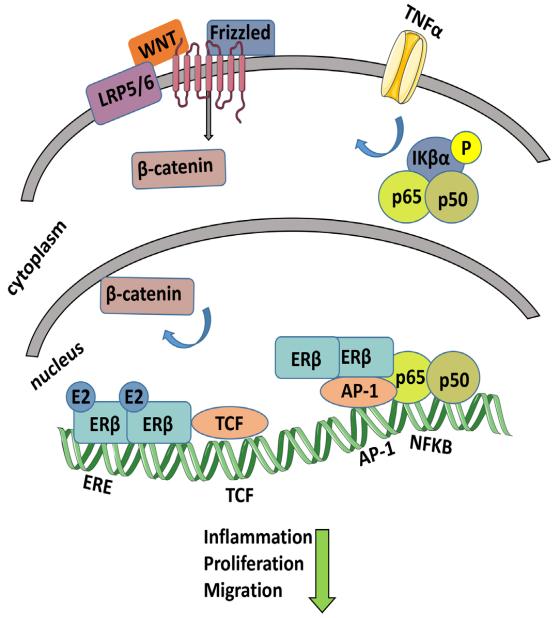

Figure 9. Schematic summary model of ERβ genomic binding and cross-talk with potential transcriptional factors such as p65, AP-1 and TCF motifs and the regulated genes are involved in inflammatory and cellular processes which are important in CRC formation and development.

In conclusion, our data indicate that intestinal ERβ is protective against CA-CRC in a sex-dependent manner by attenuating the TNFα-NFκB pathway. Through genome-wide binding studies and transcriptomic studies, we demonstrate that ERβ regulates several tumor suppressors and oncogenes involved in CRC progression. We identified that ERβ impacts the p65 cistrome and, in particular, regulates circadian clock genes and modulates p65 chromatin binding to circadian clock genes. Estrogen through ERβ also protects against HFD-induced deregulation and impacts epithelial cell proliferation and macrophage infiltration in a sex-dependent manner.

5 CONCLUSIONS

The following are the major findings of this research:

- Loss of intestinal ERβ enhances CRC in a sex-dependent manner, increasing the incidence of tumors in males and tumor size in females. This suggests that ERβ, which is present in the intestinal epithelial cells, is protective against CRC.
- ERβ attenuates TNFα-induced epithelial cell damage and reduces inflammation, as well as impacts intestinal crypt cell proliferation.
- In colon cancer cells, ERβ binds and regulates several important tumor suppressors and oncogenes involved in the oncogenic Wnt/β-catenin pathway and NFκB signaling. Novel major motifs identified in ERβ chromatin binding sites include TCF and KLF motifs. This indicates potential genomic cross-talk between ERβ and TCF and KLF factors.
- Estrogen via ERβ protects against the HFD-induced pro-inflammatory condition. Selective activation of ERβ with DPN reduced macrophage infiltration and epithelial cell proliferation. Intestinal ERβ restored the HFD dysregulated clock genes expression *in vivo*.
- ERβ attenuates the TNFα transcriptome in CRC cell lines and modifies p65 chromatin binding in a cell line-dependent manner. It also modulates the expression of NFκB target genes. ERβ impacts p65 chromatin binding to core clock genes in CRC cell lines.
- The circadian clock is essential to maintain gut function, inflammation, and metabolism, and ERβ regulating this may be imperative for its protective activity. This indicates a key role for ERβ in the regulation of clock genes, providing a novel approach to study the impact of ERβ in colonic circadian rhythm.

6 FUTURE PERSPECTIVES

The studies conducted with our animal model ERβKO^{Vil} suggest that loss of intestinal ERβ aggravates CRC and enhances TNFα signaling. We recently also performed RNA-sequencing of WT and ERβKO^{Vil} colon to investigate the protective effects of ERβ. In the future, it would be interesting to perform both AOM-DSS and HFD studies in a recently developed novel transgenic mouse model Gt(ROSA)26Sor with ERβ overexpression (iERβ+). Endogenous ERβ expression is very low in the colon, and it would be fascinating to compare the results with the ERβ knock-in model. This would help advance our understanding of the localized impact of intestinal ERβ on colon microenvironment during colitis and HFD-induced obesity. Future studies should also focus on the effects of restoring ERβ expression in adenomas (pre-stage of CRC) using an inducible intestinal ERβ mouse model. This would add to our understanding of whether restoring ERβ in tumors could exert anti-tumorigenic effects. Further, investigating approaches to restore ERβ expression in the advanced stages of CRC, as has been reported in polyps by certain phytoestrogenic/lignan complexes. Several doxycycline-inducible mouse models available are not suitable for colon studies, as doxycycline impacts the gut microbiome by decreasing bacterial diversity, richness (346), and metabolism (347). Developing safe inducible intestinal ERβ mouse models could enable us to further explore ERβ activity in detail.

My cistrome data identified novel evidence of interaction between ERβ and TCF and KLF motifs. More insight into this cross-talk can be achieved from CO-IP or possibly IP-MS. Recent methodological developments such as RIME (rapid immunoprecipitation mass spectrometry of endogenous proteins) or quantitative multiplexed RIME methods further help to explore protein interactions in complex chromatin heterogeneity (348, 349). Simultaneously, exploring ERβ interaction with AP1 or nuclear receptors expressed in colon is also essential to target NR cross-talk. In the future, this would help to design ligands to improve or restrict transcription factor cross-talk. So far, due to low levels of ERβ expression in the colon, it is challenging to perform ChIP-Seq using endogenous expression of ERβ. The iERβ+ mouse would also provide an opportunity to further corroborate the ChIP-Seq results *in vivo*.

In this study, we sequenced E2-activated ERβ ChIP samples. However, we also demonstrated (at a few selected sites) that the DNA binding of ERβ is ligand-independent in these cell lines. The reason for this is not known. In COS1 cells, it has been shown that EGF (epidermal growth factor) induces phosphorylation of serine residues (S104, S124) of ERβ and activates its interaction with steroidal co-receptor activator 1 (SRC-1), and this results in ligand-independent activity (350, 351). However, we cannot rule out the possibility that the E2 independence results from ERβ overexpression. In this regard, it is also interesting to perform ChIP-Seq without E2 ligand and compare both cistromes to determine if there is any difference present, in some or a proportion of binding sites, between ligand-activated ERβ and ligand-independent ERβ binding patterns.

In the HFD study, we have studied how ERβ impacts HFD-induced dysregulation of clock genes at only one time point. Further analyzing this at different Zeitgeber time points throughout the day will help to identify how ERβ affects circadian rhythmicity. We observed substantial sex differences in the colon transcriptome of mice. Another study from our group recently identified sexual dimorphism in humans in normal colon and colon cancer transcriptomes and presented sex-specific prognostic CRC biomarkers (352). Such sex differences may help to explain the difference in CRC incidence, tumor characteristics, and mortality between men and women. Altogether, it appears imperative that future studies of CRC and colon consider sex in their design.

My Ph.D. studies have made an interesting finding that p65 binding to circadian clock genes in colon cancer cells. More insights into this regulation could help to understand this novel finding. I have revealed that the ERβ impact on p65 activity is cell-type dependent. Also, our data shows an interesting feedback loop mechanism in which TNFα transactivates ERβ, which in turn inhibits TNFα signaling. Therefore, further research into TNFα-P65-ERβ cross-talk, *in vitro* and *in vivo,* is required.

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